Université catholique de Louvain
Faculté d'ingénierie biologique, agronomique et environnementale
Unité de phytopathologie

Diversity of soil-borne sugar beet viruses in Iran

A comprehensive study of *Beet necrotic yellow vein virus*, *Beet black scorch virus* and other pomoviruses in Iran

Mohsen Mehrvar

Thèse présentée en vue de l'obtention du grade de docteur en sciences agronomiques et ingénierie biologique
Octobre 2009

MEMBRES DU JURY

Président:	Prof. Jacques Mahillon (MBLA, UCL)
Promoteur:	Prof. Claude Bragard (FYMY, UCL)
Lecteures:	Prof. Olivier Lemaire (INRA, France) Prof. Jean François Ledent (ECAV, UCL) Prof. Anne Legrève (FYMY, UCL)

Acknowledgements

I am grateful to all of the people who supported me in this research particularly I would like to gratefully acknowledge Prof. Claude Bragard who accepted me in his lab and opened in my mind a new window of wisdom and insight toward molecular virology, enthusiastically supervised me in all moments of my PhD career in UCL and supported the project in every way possible at every stage.

I would like to express my special thanks to the member of my jury, Jean François Ledent, Olivier Lemaire, Anne Legrève and Jacques Mahillon, for their constructive advices to improve the manuscript of this thesis. Thanks for their kindness and support.

Also I should thank Prof. Henri Maraite for his support and kindness. My thanks to my dear friends Francois Crutzen, Benjamin Dieryck and Gaetan Otto, Nicolas Dedsoignies and Lu Quen for everything they did for me. Also I would like to thank all of the people in the UCL phytopathology lab for their supports, kindness and hospitality.

I would also like to thank all of teachers and professors of mine since the elementary school to date for their efforts, support and kindness.

And finally thanks to my wife who always encourages me and my parents, sisters and brothers particularly my father who encouraged me and supported me since I was a child and always motivates me to be patient and also at this research project he was so helpful to me in assisting for gathering samples in a vast country like Iran.

And at the end to my little Sonia who motivates me to work hard and hard.

TABLE OF CONTENTS

Abbreviations

aa	amino acid
AMV	*Alfalfa mosaic virus*
AGO	Argonaute protein
A-type	BNYVV isolate displaying a typical RNA composition - A-types are mainly occurring in southern, western and Eastern Europe, as well as in the Northern America
BBNV	*Broad bean necrotic virus*
BBSV	*Beet black scorch virus*
BMYV	*Beet mild yellowing virus*
BNYVV	*Beet necrotic yellow vein virus*
BSBMV	*Beet soil-borne mosaic virus*
BSBV	*Beet soil-borne virus*
BTE	*Barley yellow dwarf virus* (BYDV)-like cap-independent translation element
B-type	BNYVV isolate displaying a typical RNA composition B-types are mainly occurring in central Europe (Germany, Austria, Switzerland)
BVQ	*Beet virus Q*
BYV	*Beet yellows virus*
cDNA	complementary DNA
CP	coat protein
Ct	cycle threshold
cv	cultivar
CuNV	*Cucumber necrosis virus*
CyRSV	*Cymbidium ringspot virus*
DNA	deoxyribonucleic acid
DEPC	di-ethylpyrocarbonate
dNTP	desoxy-nucleotide triphosphate
GPS	global positioning system
dpi	days post-inoculation
dsRNA	double stranded RNA
ELISA	enzyme linked immunosorbent assay
ICTV	International Committee on Taxonomy of Viruses
IPPO	Iranian plant protection organization
J-type	BNYVV isolate containing similar to the French P-type an additional RNA5 and differing from the P-type by the truncation of four amino acids – J-types commonly occur in Asia
kb	kilo base
kDa	kilo Dalton
LWSV	*Leek white strip virus*
M-MLV RT	Moloney-Murine leukaemia virus reverse transcriptase
MP	movement protein

mRNA	messenger RNA
NC	negative control
NES	nuclear export signal
NLS	nuclear localization signal
nt	nucleotide
OLV	*Olive latent virus*
OMMV	*Olive mild mosaic virus*
ORF	open reading frame
P	protein
PB	*Polymyxa betae*
PC	positive control
PCR	polymerase chain reaction
PD	plasmodesmata
PMTV	*Potato mop-top virus*
PoLV	*Pothos latent virus*
PTGS	post-transcriptional gene silencing
P-type	BNYVV isolate displaying a typical RNA composition and sometime additional fifth RNA, P-types are mainly occurring in a small region in France (Pithiviers), the UK and in Kazakhstan
RB	resistance-breaking
RdRp	RNA dependent RNA polymerase
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
Rz1	resistance gene against BNYVV from the “Holly” source
Rz2	resistance gene against BNYVV from the WB42 source
Rz3	resistance gene against BNYVV from the WB41 source
Sat	satellite
sgRNA	subgenomic RNA
siRNA	short interfering RNA
SSCP	single strand confirmation polymorphism
STNV	*Satellite tobacco necrosis virus*
TBSV	*Tomato bushy stunt virus*
TCV	*Turnip crinckle virus*
TE	translation element
TGB	triple gene block
TM	transmembrane
TMV	*Tobacco mosaic virus*
TNV	*Tobacco necrosis virus*
TPIA	tissue print immunoassay
UTR	untranslated region

Chapter I. Introduction

Introduction

In this thesis, the distribution, level of diversity and impact of soil-borne viruses on sugar beet in Iran will be addressed. Briefly, sugar beet has been known for a long time in the country (Sohi & Maleki, 2004; Farzadfar *et al.*, 2007). Recently, several soil-borne viruses have been discovered. Nevertheless, until now, there was a lack of information regarding their distribution, genetic diversity and real impact on the sugar beet crop.
With the view of addressing this issue, this thesis is planning to unravel the complex situation of soil-borne viruses in Iran, by studying four different viruses: the *Beet necrotic yellow vein virus*, a *Benyvirus*, the *Beet soil-borne virus* and the *Beet virus Q*, two *Pomovirus*, and finally the recently discovered *Beet black scorch virus*, a *Necrovirus* along with its satellite, the *Beet black scorch virus* satellite. The three first viruses are telluric viruses transmitted by the plasmodiophorid vector *Polymyxa betae*, while the BBSV and its satellite are transmitted by the Chytrid *Olpidium brassicae*.
The crop of sugar beet in Iran, the different soil-borne viruses and their vectors will be shortly described in an introduction before presenting and discussing the thesis results.

I.1. Sugar beet in Iran

The genus *Beta* is native to Eastern mediterranean region and adjacent areas. In the ancient times beets were cultivated in the Mediterranean countries (Lennefors, 2007). Section *Nanae* (Greece) and *Procumbentes* (Canary Island) have a limited distribution area, while wild species of section *Beta* occur along the coastline from the south of Sweden to Morocco and from the canary Island to Iran. Section *Corollinae* occurs at altitudes higher than 800 m. It has a large distribution area in Turkey and neighbouring countries. The center of diversity is probably located where the species distribution of sections *Beta* and *Corollinae* overlap (eastern Turkey and the western part of Transcaucasia) (Frese *et al.*, 2001). The domestication of beets probably started in the Euphrates and Tigris region and continued in Turkey and Greece, from where cultivated beets were introduced to northern Europe (Boughey, 1981; Frese *et al.*, 2001).
In Europe, cultivated forms of *Beta* (leaf beet, garden beet, fodder and sugar beet) are grown on more than 5.6 million ha, to which can be added approximately 550,000 ha in North America; 600,000-670,000 ha in China (with a promising production and market potential for sugar beet); and a number of smaller areas in Chile, Egypt, Iran, India, Japan, Morocco, Tunisia, Turkey and Syria. Altogether, sugar beet are grown on about 7.5-8.1 million ha in 41 countries worldwide.

Breeding efforts are focused on the sugar beet crop, while leaf, garden and fodder beet breeding is of regional importance only (Rush *et al.*, 2006). Sugar beet is a key production for agriculture in many of countries across the world. It provides more than 25% of the world's sugar supply (Rush *et al.*, 2006).
In Iran roughly one-third of total surface area of the country is suited for farmland, but because of poor soil and lack of adequate water distribution in many areas, most of it is not under cultivation. Presently only 12% of the total land area is cultivated (arable land, orchards and vineyards) but less than one-third of the cultivated area is irrigated; the rest is devoted to dry farming. The Northeastern, Western and Northwestern portions of the country have the most fertile soils and under the cultivation of agricultural crop e.g sugar beet which estimates of 180,000 hectares (Fig. 1). Constraints in sugar beet production in Iran are droughtness as well as pest and disease which limit the production in the country. From viral sugar beet disease the soil-borne viruses are the most threat for Iranian sugar beet industry specially BNYVV the causal agent of rhizomania disease which presents nearly in most of the sugar beet cultivation areas and cause significant damages to sugar beet crop annually (Farzadfar *et al.*, 2007).

	Provinces	Surface under sugar beet cultivation (2005 -2006) Ha
1	Azarbaeijan Gharbi (9)	33607
2	Azarbaeijan Sharghi (8)	340
3	Ardabil (6)	4461
4	Esfahan (24)	6976
5	Fars (19)	22310
6	Ghazvine (4)	3647.5
7	Hamadan (11)	10825
8	Kerman (22)	3097.5
9	Kermanshah (12)	15535
10	Khorasan Jonobi (30)	7838.1
11	Khorasan Razavi (29)	51089
12	Khorasan Shomali (28)	4352
13	Semnan (25)	6265
14	Others (4, 13)	15544.6
Total		185887.7

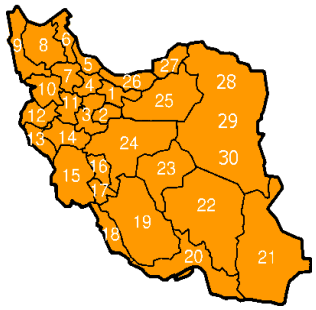


Fig. 1 Surfaces which were under cultivation of sugar beet in 2005-2006 (**left**) and map of different provinces in Iran (**right**). The number in the parenthesis indicates the provinces on the map (http://www.sbsi.ir/default_e.asp).

I.2. Soil-borne sugar beet viruses vectored by *Polymyxa betae* KESKIN

Beet necrotic yellow vein virus (BNYVV), *Beet soil-borne virus* (BSBS), *Beet virus Q* (BVQ) and *Beet soil-borne mosaic virus* (BSBMV) are four different soil-borne sugar beet viruses which are transmitted by *Polymyxa betae* a plamodiophorid. The BNYVV is the causal agent of most destructive viral disease of sugar beet the rhizomania. BSBV and BVQ which are two pomoviruses are usually associated with BNYVV. BSBMV is sometime present with BNYVV in USA. BSBMV has not been reported from other country.

I.2.1. *Beet necrotic yellow vein virus*

I.2.1.1 *Beet necrotic yellow vein virus* the causal agent of rhizomania disease

The rhizomania disease was first detected in the early 50s in northern Italy in the Po valley (Canova, 1959), and now is found in most of the sugar beet growing areas of the world including the USA, Japan, China, Russia, Europe and the Middle East (Asher, 1993; Rush, 2003; McGrann *et al.*, 2009).
In Iran it was first reported from the Fars province in 1996, it is now found in nearly all sugar beet growing areas of the country and causing huge damages to the crop (Sohi & Maleki, 2004; Farzadfar *et al.*, 2007).
The disease has caused severe economic losses in all infected regions. The name of the disease is derived from one of its typical symptom, which is an intense rootlets proliferation (Fig. 2). The disease is characterized by a very efficient rate of propagation. For example, the infected surface in the United-States has increased from zero to more than 30000 hectares from 1983 to 1988 (Harveson *et al.*, 1996; Asher, 1999). In 2000, the percentage of affected fields is of 46% in France, 70% in The Netherlands and 35% in Germany (Giltrap *et al.*, 2001). Irrigation (Tuitert & Hofmeester, 1992) but mainly movement of soil during tillage and harvest are responsible for the spread of the vector and virus (Harveson *et al.*, 1996). Breeding for resistance is by now, only efficient control measure and partially rhizomania-resistance sugar beet cultivars are available which reduce yield losses in infected areas. However, no cultivars are completely resistant. Recently sugar beet genotypes carrying one (*Rz1*) as well as two resistance genes (*Rz1+Rz2*) showed some kind of resistance breaking in Spain and US and caused a great concern (Koenig *et al.*, 2005; Acosta-Leal & Rush, 2007). The disease can lead to a severe decrease of yield (Asher, 1999).

The disease affects the sugar yield by diminishing the root weight and decreasing the sugar content of the sugar beet (Asher, 1999). The typical symptoms allowing the identified of the rhizomania disease are observed both at the root and leaves (Fig. 2) with characterized tap root constriction, profuse lateral root proliferation, and leaf chlorosis (Acosta-Leal *et al.*, 2008). A recent proteomic analysis of infected sugar beet by BNYVV has shown an induction of phytohormones, such as abscisic acid and auxin, which may be involved in the production of the 'root madness' symptoms characteristic of rhizomania (Larson *et al.*, 2008) (Fig. 2). The rootlets system is dense having a bear-like appearance. Constriction and pale brown coloration of the vessels can also be observed at the extremity of the sugar beet tap root resulting in the necrosis of the tissues, which turn brown and finally may be dying. The foliar symptoms often caused as a result of root vascular damages and reduced nutrient uptake but rarely the BNYVV infection becomes systemic and the leaves show a variety of symptoms, including yellowing, crinkling, wilting and vein yellowing that gives the virus its name (McGrann *et al.*, 2009), and leaves sometimes have longer petiole, light-yellow color, and narrow limb. The presence of the disease will be accompanied in the field by the presence of light green or yellow irregularly patches or yellow rows along side of furrows in sugar beet fields which is the first sign of rhizomania disease usually during the early months of the growing season (Fig. 2E) (McGrann *et al.*, 2009). Rarely the yellowing of the leaves evolve to necrosis of the veins. Sugar beet plants infected by rhizomania have low sugar concentration, high sodium and low alpha-amino nitrogen contents and these parameters reduce the storage ability of roots, resulting in further losses to the crop (de Biaggi, 1987; Heijbroek, 1989; Asher, 1993; Krabovic & Kralova, 1996; Wauters *et al.*, 1997; Strausbaugh *et al.*, 2008). The lower sugar content which reduce between 8%-48% combined with the decrease of the root weight sometime nearly 50% lead to important yield losses up to 50%-60% although losses of almost 80% have been reported as well (Merdinoglu *et al.*, 1993; Asher, 1999; Tamada *et al.*, 1999; McGrann *et al.*, 2009).

In order to detect the disease, different tests are performed in the labs and sugar refineries, before it was detected in plant by use of ELISA technique (Koenig *et al.*, 1984; Torrance *et al.*, 1988; Wisler *et al.*, 1999; Uhde *et al.*, 2000). However more sensitive technique of RT-PCR and nested RT-PCR can also be used (Henry *et al.*, 1986; Morris *et al.*, 2001; Meunier *et al.*, 2003). This technique allows the detection of the BNYVV in bait plants only two weeks after contamination compared to three to four weeks for ELISA.

The quantative real-time RT-PCR is now used for measuring the titer of the virus inside the host plants and detection of the virus at every

stage of sugar beet infections (Harju *et al.*, 2005). Meunier *et al.* (2003) have shown that the multiplex detection of BNYVV, BSBV, BVQ and PB is possible. Ratti *et al.* (2005) reported the detection of two types of A and B of BNYVV by a multiplex RT-PCR, using primers targeting TGB (triple gene block) region of BNYVV RNA2.

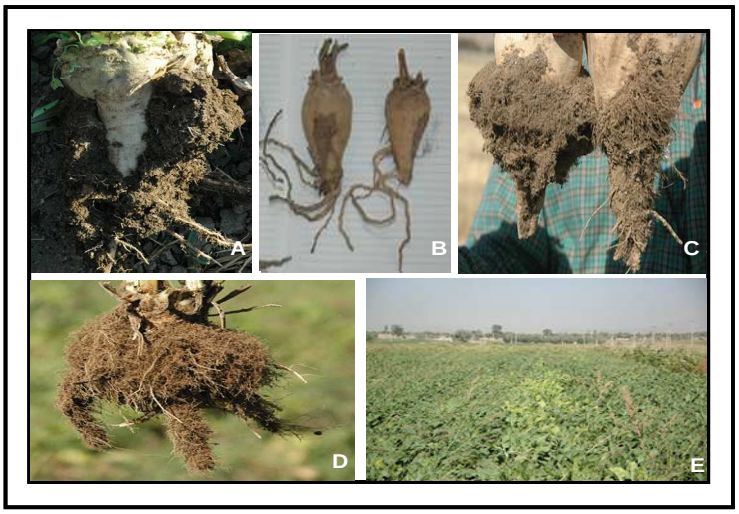


Fig. 2 (A) Bearded shape of infected sugar beet roots by BNYVV in different parts of Iran (Nyshabour, Khorasan), **(B)** (Kerman), **(C)** (Malayer, Hamadan) **(D)** (Asad Abad, Hamadan), **(E)** Yellowing of sugar beet along side of furrows in a sugar beet field infected by BNYVV in Iran (Malayer, Hamadan)

I.2.1.2 *Beet necrotic yellow vein virus* genome organization

Beet necrotic yellow vein virus was identified in the 70s (Tamada & Baba, 1973). It is a member of genus *Benyvirus*, which also include the *Beet soil-borne mosaic virus* (BSBMV), however these genera have not yet been assigned to a family. These viruses are characterized by their ability of transmission to the plant by *Plasmodiophoridae*, by their rigid rod shaped morphology and by their multipartite genome. In the case of BNYVV, the vector is *Polymyxa betae* KESKIN (Rush, 2003).

BNYVV is a single-stranded RNA virus with tubular rod-shaped particles. In the rootlets of BNYVV infected sugar beet, four RNA particles are invariably present (Koenig *et al.*, 1997b). The four BNYVV particles have a diameter of 20 nm and a length of 390 (RNA-1), 270 (RNA-2), 105 (RNA-3) and 90 nm (RNA-4). A fifth

RNA molecule of 80 nm length is present in some isolate from Asia and Europe (Tamada, 1975; Putz, 1977; Tamada & Abe, 1989; Koenig & Lennefors, 2000; Ward *et al.*, 2007). All five RNAs possess a cap (m^7GpppG) at the 5′ extremity and are poly-adenylated (poly(A)) at the 3′ terminus and these segments are involved in replication and regulation of translation as eukaryotic translation systems (Fig. 3) (Richards & Tamada, 1992; Hull, 2002; Mandahar, 2006).

RNA-1 and RNA-2 have 'housekeeping' genes which are involved in basic viral cycle such as replication, encapsidation and cell to cell movement while RNA-3 –4 and –5 are associated in vector-mediated infection and development of the disease (Lemaire *et al.*, 1988; Richards & Tamada, 1992) (Fig. 3). In sugar beet protoplasts infections, the virus particles are detected already after 16 hours with a maximum reached after three days (Joersbo & Brunstedt, 1990). Cooper and Asher (1988) have reported the completion of BNYVV cycle in sugar beet within 48 hours.

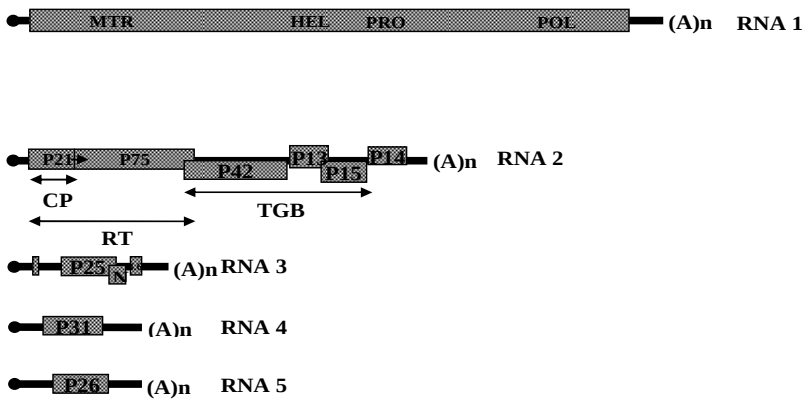


Fig. 3 The genome organization of *Beet necrotic yellow vein virus*. The filled circles indicate the cap structures, the boxes indicate ORFs, and the (A)n indicates conserved common sequences preceding the 3'-poly (A) tail. The internal arrow in RNA2 indicates a readthrough amber termination codon. The coat protein (CP), the readthrough protein (RT) and the triple gene block (TGB) are shown by arrows. The methyltransferase domain (MTR), the NTP-binding/helicase domains (HEL), the papain-like proteinase domain (PRO), the GDD replicase domain (POL) are shown on RNA1 coding region.

RNA-1 contain one large open reading (ORF) that is 6746 nucleotides long, encoding for p237, a putative replication-associated polypeptide protein (Jupin *et al.*, 1988), which is post-translationally cleaved due

to the presence of a papain-like protease motif into two proteins of 150 and 66 kDa (Bouzoubaa *et al.*, 1987; Tamada *et al.*, 1999), These products are involved in viral replication and possess replication-associated domains: a methyltransferase domain (MET), an NTP/helicase domain (HEL) and a polymerase domain (POL), whereas polymerase domain is found within p66 (Richards & Tamada, 1992; Hehn *et al.*, 1997). This is a unique character of benyviruses from all other plant viruses with rod shaped particles, which have their replication-associated proteins encoded on two ORFs (Fauquet *et al.*, 2005).

Scientists showed that the organization of the domain of p237 and polymerase and helicase sequences were part of the "alpha-like" supergroup grouping HEV, Rubv and BNYVV. RNA-1 alone is capable of auto replication, indicating that it contains all the information needed for replication (Koonin *et al.*, 1992; Tamada *et al.*, 1999).

RNA-2 is 4612 nucleotides long and contain six open reading frames (Fig. 3) (Bouzoubaa *et al.*, 1986). This RNA encoded for major (p21) (Putz, 1977) and minor (p75) capsid proteins which encodes by a readthrough (RT) mechanism in its proximal 5' ORF through a 'leaky' amber termination. Different epitopes were identified on p21 capsid protein (Koenig *et al.*, 1990; Commandeur *et al.*, 1992; Schmitt *et al.*, 1992; Commandeur *et al.*, 1994; Meunier, 2005).

p75 plays important roles in vector transmission (Tamada & Kusume, 1991) with two important transmembrane domains, found in other viruses transmitted by *Polymyxa* and *Spongospora* that could interact with *P. betae*'s membrane (Dessens & Meyer, 1996; Adams *et al.*, 2001). The important "KTER" (Lys. Thr. Glu & Arg)" motif is located in one of these transmembrane domains (Tamada & Kusume, 1991; Tamada *et al.*, 1996b; Koenig, 2000). p75, which is detected at the ends of viral particles, is also required for virus encapsidation process. Therefore, p21 and p75 will be invariably detected in all particles regardless of which of the five BNYVV RNAs are encapsidated (Schmitt *et al.*, 1992; Haeberle *et al.*, 1994). It is thought that these genomic regions of plasmodiophorid-transmitted viruses encode transmembrane domains that are involved in attachment to the vector plasmalemma, help the virion transmission between plant and vector cytoplasm (Tamada & Kusume, 1991; Koenig, 2000; Adams *et al.*, 2001; Meunier, 2005). p75 also appears to be involved in targeting the virus assembly to the mitochondria of host cells (Erhardt *et al.*, 2001; Valentin *et al.*, 2005).

RNA-2 also gives rise to two subgenomic RNAs (sgRNAs). Such RNAs are expressed from internal promoters located on complementary minus strand and located on certain open reading frame. These sgRNAs encode for proteins involved in the cell to cell

movement. These proteins composing the "triple gene block" (TGB) are named p42, p13 and p15 (Haeberle *et al.*, 1994; Hehn *et al.*, 1994; Bleykasten-Grosshans *et al.*, 1997; Lauber *et al.*, 1998). p42 is produced by mean of a monocistronic subgenomic RNA while p13 and p15 production is directed by a dicistronic subgenomic RNA and these TGB ORFs are slightly overlapped (Gilmer *et al.*, 1992a; Fauquet *et al.*, 2005). Expression of the first two TGB proteins can complement independently, but over expression of last one (15 kDa) inhibits cell to cell movement of the virus infection (Tamada *et al.*, 1999). p42 has motifs characteristic of helicase and nucleic acids binding ("P-loop"ATP/GTP binding domain) (Koonin & Dolja, 1993; Bleykasten-Grosshans *et al.*, 1997). p13 has two hydrophobic domains separated by a hydrophilic sequence. p15 is also hydrophobic. The p13 and p15 proteins both localise to cell wall thickenings that are believed to represent degenerated plasmodesmata (Erhardt *et al.*, 2005). Both proteins are required *in planta* to target p42 to punctate bodies that are associated with the plasmodesmata to enable virus movement between cells (McGrann *et al.*, 2009).

At the 3′ end of RNA-2 exists an ORF giving protein p14 which is implicated in control of RNA-2 accumulation (Hehn *et al.*, 1995) but also could act as a post transcriptional gene silencing (PTGS) inhibitor (Dunoyer *et al.*, 2002). This protein shows *in-vitro* zinc ions binding activity suggesting nucleic acid binding activity (Dunoyer *et al.*, 2002), it also acts in *trans* as an enhancer of viral coat protein synthesis (Tamada *et al.*, 1999).

RNA-3 consists of 1775 nucleotides and encodes a 25-kDa protein (p25), which has been shown to modify foliar symptoms expression in particular host plants following inoculation of the leaves (McGrann *et al.*, 2009) (Fig. 3). It is responsible for symptoms expression and also required for the proliferation of fibrous roots during natural infection of sugar beet (Koenig *et al.*, 1991; Tamada *et al.*, 1999). Deletion occurring in the p25 ORF leads to decrease of symptoms expression (Tamada *et al.*, 1999). This RNA is also involved in long distance movement in *Beta macrocarpa* (Tamada & Abe, 1989) and facilitates the spread of the virus in the plant (Koenig & Ehlers, 1989). Therefore, differences within this RNA or of the proteins it encodes could involve differences in term of virulence. Based on these findings, it has been suggested that RNA3 is a pathogenicity determinant of BNYVV and that p25 is responsible for the symptoms of rhizomania in sugar beet (Jupin *et al.*, 1991; Chiba *et al.*, 2008; McGrann *et al.*, 2009).

Severe necrotic symptoms appear to be caused following mechanical inoculation of leaves when the p25 protein is able to access both the cytoplasm and nuclear compartment of the host cells (Vetter *et al.*, 2004).

p25 is soluble *in vivo* (Niesbach-Klosgen *et al.*, 1990) and was detected in both cytoplasm and nuclei in infected leaves (Haeberle & Stussi-Garaud, 1995; Li *et al.*, 1996). The localization in the nuclei, the existence of a potential "zinc finger" motif (residues 73 to 90) (Berg, 1990), the presence of a nuclear localization/exportation signals (NLS and NES) (Vetter *et al.*, 2004) and evidence that p25 can fix zinc ions (Niesbach-Klosgen *et al.*, 1990) suggest an interaction with the host genome, maybe acting as a transcription factor (Jupin *et al.*, 1992; Haeberle & Stussi-Garaud, 1995).

Several p25 variants exist within natural isolates and p25 sequence variation may influence the degree of pathogenicity of such BNYVV isolates. A phylogenetic approach permitted the division of BNYVV isolates into subgroups that mainly differ by the p25 sequence within a variable motif named the tetrad, that is present directly downstream of the p25 NLS sequence. Schirmer *et al.* (2005) analyzed the genetic variability of 136 viral isolates collected worldwide and found that the RNA 2 encoding CP cistron is highly conserved, whereas p25 (RNA 3) and p26 (RNA 5) genes are more variable with strong positive selection acting on some of their amino acids. The p25 and p26 genes operate synergistically to exacerbate symptoms in certain sugar beet cultivars; they localize in the nucleus and activate transcription (Schirmer *et al.*, 2005). Klein *et al.* (2007) did express of p25 from natural A-and P-type isolates in the background of B-type BNYV cDNA clones, showing symptom discrepancies in comparison to B-type expression and concluded that such pathogenicity fluctuation was not due to a different subcellular localization of p25 but was correlated with the nature of the tetrad motif present between amino acid residues 67-70, as well as with capacity of p25 to self-associated and to activate transcription in a yeast one-hybrid system also they concluded that the complete sequence of p25 is required for its functions and the identified sequence variations may contribute to correct folding of the protein (Klein *et al.*, 2007). Furthermore, recent evidence has suggested that the p25 protein may act as the avirulence target recognised in the mechanically inoculated leaves of some BNYVV-resistant sugar beet lines (Chiba *et al.*, 2008).

Sequences important for RNA-3 replication were localized in the 70 residue of the 3′ non coding region (Lauber *et al.*, 1997). Cis active sequences forming hairpins and required for replication and also containing an encapsidation signal are present in the first 300 nt. at the 5′ extremity of RNA-3 and –4 (Gilmer *et al.*, 1992b; Gilmer *et al.*, 1993). Recently scientists shown that p25 and p31 proteins accumulate to significant levels in *P. betae* and localize to the zoospore nucleus (Lubicz *et al.*, 2007) .

In RNA-3 also three little ORFs have been reported: ORF A, ORF N and ORF 4.6 (or S). p4.6 is produced from a subgenomic RNA. Its

promoter is mapped at nt. 1230 (Balmori *et al.*, 1993) but the function of p4.6 is unknown (Bouzoubaa *et al.*, 1991). Serial mechanical inoculation of BNYVV on *Chenopodium quinoa* has led to the occurrence of deleted form of RNA-3 and RNA-4. ORF N overlaps the C terminus of p25 ORF. This "N" protein is thought to be responsible for important necrosis symptoms when inoculated on leaves but is only expressed when activated by deletions of upstream sequences arise in p25 ORF or when inserted into another plant virus replicon (Jupin *et al.*, 1991; Jupin *et al.*, 1992; Tamada *et al.*, 1999).RNA-4 is 1413 nucleotide long and contains two ORFs (Bouzoubaa *et al.*, 1985) (Fig. 3). The major ORF encodes a 31-kDa protein (p31), which is required for efficient transmission of the virus by *P. betae* (Tamada & Abe, 1989), the other ORF codes for a 6.5-kDa protein of unknown function, although it is unknown whether its protein product, p31, or replication of the RNA4 sequence is required for enhanced transmission efficiency. p31 has also been implicated in other infection-related processes, such as the enhancement of BNYVV symptom expression, in hosts such as *Nicotiana benthamiana* where RNA4 rather than RNA3 has been shown to be associated with severe symptom development via BNYVV mediated suppression of RNA silencing in roots (Rahim *et al.*, 2007; McGrann *et al.*, 2009). RNA4 is not essential for virus multiplication and produce strong chlorotic lesions in *Tetragonia expansa* leaves in absences of RNA3, whilst those lacking RNA4 produced only faint chlorotic lesions (Tamada & Abe, 1989; Richards & Tamada, 1992). Andika *et al.* (2006) suggested that roots have less of an activity that acts at the step of generation of siRNAs, and later Rahim *et al.* (2007) found that RNA4 p31 plays a multifunctional role in efficient vector transmission, enhanced symptom expression and root-specific silencing suppression activities.

Specific isolates of BNYVV hold a supplementary RNA-5. RNA-5 has 1342-1347 nucleotides and encodes just one protein called p26. It is observed in sources originating from Japan, China, France, United Kingdom and Kazakhstan (Kiguchi *et al.*, 1996; Koenig & Lennefors, 2000; Harju *et al.*, 2002; Ward *et al.*, 2007), is thought to be responsible of even more severe rhizomania symptoms (Tamada *et al.*, 1996a). The single polypeptide it encodes (p26), differs between J- and P–type isolates and exacerbates symptom severity in synergy with RNA3 (Schirmer *et al.*, 2005; Acosta-Leal & Rush, 2007). Also Link *et al.* (2005) found that the entire p26 protein rather than a specific domain of it is required for induction of necrosis local lesions in *C. quinoa* and appears to be partially targeted to the nuclear compartment of infected host cells.

I.2.1.3 Different types of BNYVV

Based on molecular analyses of the four RNA species which is always present in natural BNYVV infections, have revealed the existence of three major genotypes of BNYVV in the world; named A, B and P types. These types were originally recognized by means of restriction fragment length polymorphism (RFLP) and single-stranded conformation polymorphism (SSCP) analysis in BNYVV RNA1, RNA2, RNA3 and RNA4 as well as by phylogenetic relationships (Kruse *et al.*, 1994; Koenig *et al.*, 1995; Schirmer *et al.*, 2005; Ward *et al.*, 2007). The RFLP patterns of the B type correspond to the sequences published by Bouzoubaa *et al.* (Bouzoubaa *et al.*, 1985; Bouzoubaa *et al.*, 1986; Bouzoubaa *et al.*, 1987) for the French isolate F2, whereas those of the A type are predicted from the sequences of their isolate G1 for which only deletion mutants of RNAs 3 and 4 have been analyzed. More detailed information on the sequence differences between European A, B and P types has been given by Koenig and Lennefors (2000) and Ward *et al.* (2007), accession numbers for reference sequences are listed in the VIIIth report of the International Committee on Taxonomy of Viruses (Koenig & Lennefors, 2000; Fauquet *et al.*, 2005; Ward *et al.*, 2007). A and B types are most common types of BNYVV in which the A-type has a worldwide distribution whereas the B-type appears to be more restricted in its distribution, and is found mainly in France, Germany, Japan and Iran. However, the B-type has also been reported in other countries, such as the UK and Sweden (Koenig *et al.*, 1995; Miyanishi *et al.*, 1999; Koenig & Lennefors, 2000; Lennefors *et al.*, 2000; Sohi & Maleki, 2004). Type A and B have been found to be highly conserved in geographically widely separated regions. Recently determined sequences are practically identical to those determined two decades ago (Bouzoubaa *et al.*, 1985; Bouzoubaa *et al.*, 1986; Bouzoubaa *et al.*, 1987; Li *et al.*, 1999; Koenig & Lennefors, 2000; Schirmer *et al.*, 2005; Ratti *et al.*, 2006; Ward *et al.*, 2007). Differences in nucleotide sequence level between A and B types of BNYVV are 3 to 6 % (Li *et al.*, 1999; Miyanishi *et al.*, 1999) although CP of them is more conserved (Saito *et al.*, 1996; Koenig & Lennefors, 2000; Schirmer *et al.*, 2005). Ratti *et al.* (2005) reported the distinguish of type A and B in PCR bases and by use of TGB sequences which have sufficient differences to distinguish this two types from each other. BNYVV originated from Japan and China (East Asia) is similar to the European A and B types, but are not completely identical to them (Koenig *et al.*, 1991; Miyanishi *et al.*, 1999; Koenig & Lennefors, 2000; Meunier *et al.*, 2003; Meunier *et al.*, 2005; Ward *et al.*, 2007).

A distinct variant of RNA5 has been found to be associated with P type BNYVV, a virus type which so far has been found only in French, Kazakhstan and UK (Koenig *et al.*, 1997b; Koenig & Lennefors, 2000; Schirmer *et al.*, 2005). Schirmer *et al*. (2005) have distinguished the Japanese RNA5 groups and the French RNA5 holding isolates from Pithiviers due to the high sequence variability into BNYVV P and J types. In East Asian samples, variants of a fifth RNA species have frequently been found to be associated with A- or B-type-like BNYVV, also most recently Koenig *et al*. (2008) found a new A type in Germany holding RNA5 and resembling Chinese isolate Har4. Although the presence of RNA5 does not designate automatically an isolate as P-type BNYVV, RNA5 from P-type isolates characteristically shares about 96% sequence identity with RNA5 from East Asian isolates (Koenig *et al.*, 1997b; Miyanishi *et al.*, 1999; Ward *et al.*, 2007; McGrann *et al.*, 2009). Sequence comparisons of the A-, B- and P-type BNYVV isolates have suggested that the A- and P-types are more closely related than the B-type (Koenig & Lennefors, 2000; Meunier *et al.*, 2005; Ratti *et al.*, 2005; Schirmer *et al.*, 2005). It is believed that the A- and B-type BNYVV became separated a long time ago (Tamada *et al.*, 2002), and that the P-type evolved from the A-type virus. There is evidence that RNA-5-containing BNYVV sources are more pathogenic than those lacking the fifth RNA (Tamada & Baba, 1973; Lemaire *et al.*, 2003). Some studies showed that the P type has some characters like moving in host plant more rapidly, high virus titer in tolerant varieties and evading from host defense system specially in places which is cultivated by tolerant varieties which is a matter of concern (Heijbroek *et al.*, 1999; Klein *et al.*, 2007; McGrann *et al.*, 2009). Some studies however have reported the occurrence of a new kind of isolate possessing an RNA-3 molecule which is related to the severe type P but lacking the usually associated RNA-5 in the region of Pithivers and these isolates are more aggressive in resistant varieties (Lemaire *et al.*, 2003).

Appearance of severe rhizomania symptoms in partially resistant sugar beet have been reported recently in the USA where Liu *et al*. (2005) reported occurrence of resistance-breaking (RB) BNYVV in Imperial Valley in California (US) and suggested that these isolates likely evolved from existing A-type isolates. It suggested that high levels of BNYVV inoculum in the soil, coupled with environmental conditions conducive to disease development, can result in the erosion of partial rhizomania resistance (Asher *et al.*, 2002; McGrann *et al.*, 2009). These US resistance-breaking isolates were normal A-type BNYVV but there was changes in their RNA3 p25 position 67 and 68 of amino acid sequences which was AF, AL, SY, VC, VL and AC rather than AC in normal isolates (not resistance-breaking isolates) (Liu *et al.*, 2005; Schirmer *et al.*, 2005; Rush *et al.*, 2006; Liu &

Lewellen, 2007). A motif of four amino acids encompassing the same region, residues 67–70, has also been demonstrated to have a functional role in resistance breaking (Schirmer *et al.*, 2005). In particular, the residue at position 68 of the p25 protein is thought to strongly affect the accessibility of plant host factors that specifically recognize this viral protein and enable the plant to mount a defense response against the virus (Chiba *et al.*, 2008). In addition, recent analyses have indicated that there has been positive selection for these amino acid changes at positions 67–68 in resistance-breaking isolates (Acosta-Leal *et al.*, 2008). Interestingly, in the French P-type isolate, this motif is represented by SYHG, which was also found in the US isolates (Liu & Lewellen, 2007). Together, these data indicate that amino acid changes in this motif may not be a consistent indicator of the resistance-breaking potential of BNYVV isolates. However, variability within this region does appear to be associated with the breaking of resistance, and this may provide a good starting point to elucidate the mechanisms involved in resistance breaking in BNYVV (McGrann *et al.*, 2009).

I.2.1.4 Host range and symptomatology

The virus has a narrow host range. In nature, it infects sugar beet, fodder beet, Swiss chard and spinach. It is transmitted by inoculation of sap to most species of the family *Chenopodiaceae* and several species belonging to the *Aizoaceae, Amaranthaceae, Caryophyllaceae* and *Solanaceae*. Many of these plants are hosts of the vector *Polymyxa betae* (Abe & Tamada, 1986; Abe & Ui, 1986; Barr & Asher, 1992; Hugo *et al.*, 1996). The virus tends to be restricted to the inoculated leaves of host plants; it becomes systemic in *Beta macrocarpa*, some accessions of *B. vulgaris* spp. *maritima*, spinach and *Nicotiana benthamiana* (Tamada, 1975).

I.2.2. *Beet soil-borne mosaic virus*

The *Beet soil-borne mosaic virus* (BSBMV), also transmitted to the sugar beet by *Polymyxa betae* was detected only in the USA (Rush & Heidel, 1995; Heidel *et al.*, 1997). This virus and BNYVV possess a similar genome organization and identity from 20 to 90%, according to the region of the genome. Interaction between BSBMV and BNYVV was reported (Piccinni & Rush, 2000; Wisler *et al.*, 2003). It seems that BSBMV causes no damage to the plant (Rush *et al*., 1996; Lee *et al*., 2001). Ratti *et al.* (2009) reported that *Beet soil-borne mosaic virus* RNA-3 is replicated and encapsidated in the presence of

BNYVV RNA-1 and -2 and allows long distance movement in *Beta macrocarpa*.

I.2.3. *Beet soil-borne virus* and *Beet virus Q*

The *Beet soil-borne virus* (BSBV) and *Beet virus Q* (BVQ) also transmitted to the sugar beet by *P. betae*. Both of these viruses belong to *Pomovirus* and are associated with BNYVV.

I.2.3.1 Pomoviruses genome organization

Pomovirus particles are hollow, helical rods, 18–20 nm in diameter, comprising multiple copies of a single major coat protein (CP; c. 19–20 kDa). The CP gene is terminated by a UAG (or UAA in *Broad bean necrosis virus* (BBNV)) stop codon that is thought to be suppressible, readthrough (RT) of which would produce a fusion protein of variable mass (54–104 kDa). *Pomovirus* particles are fragile and particle size distribution measurements are variable; PMTV particles have predominant lengths of 125, 137, and 283 nm (Torrance, 2008).
Pomovirus genomes comprise three species of positive-sense single-stranded RNA of c. 5.8–6, 2.8–3.4, and 2.3–3.1 kbp (Fig. 4, 5).

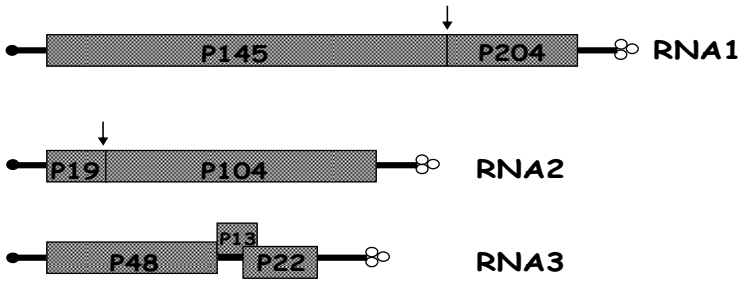


Fig. 4 BSBV genome organization

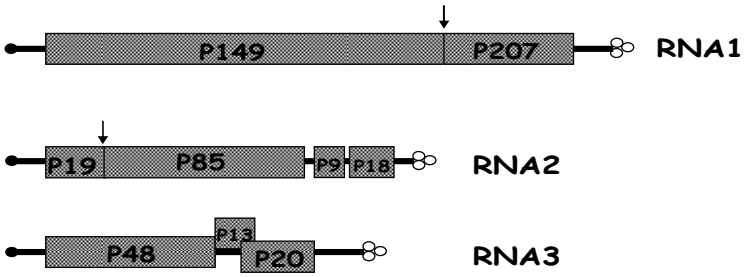


Fig. 5 BVQ genome organization

RNA1 encodes the replicase proteins. It contains a large open reading frame (ORF) that is interrupted by a UGA stop codon (ORF1) (or UAA in BVQ and BSBV (Fig. 4, 5)); the sequence continues in-phase to encode an RT protein (204–207 kDa). The ORF1 protein (145–149 kDa) contains methyltransferase and helicase motifs while the RT domain contains the GDD RNA dependent RNA polymerase (RdRp) motif. Phylogenetic analysis reveals that the RdRp's of the pomoviruses and *soil-borne wheat mosaic furovirus* share between 50% and 60% sequence identity. The 5' UTR of *Pomovirus* RNAs contains the starting sequence GU(A)1–4(U)n (except BVQ RNA1 which begins with AUA). The RNAs are probably capped at the 5' end since the RNA1 ORF contains methyltransferase motifs associated with capping activity. The terminal 80 nucleotides of the 3' UTR can be folded into a tRNA-like structure that contains an anticodon for valine. Both BSBV and PMTV RNAs were shown to be valylated experimentally. The virus movement proteins are encoded on RNA3 of BSBV (Fig 4), BVQ (Fig 5), and BBNV (RNA2 in PMTV). Three 5' overlapping ORFs encode a conserved module of movement proteins known as the triple gene block (TGB). TGB movement proteins are found in the genomes of other rod-shaped viruses (hordei-, beny, pecluviruses) and in monopartite filamentous viruses in the genera *Potexvirus* and *Carlavirus*. The TGB proteins (TGB1, TGB2, and TGB3) are named according to their position on the RNA and have molecular masses of 48–53, 13, and 20–22 kDa, respectively. The TGB1 contains a deoxyribonucleotide triphosphate (dNTP) binding site and helicase motifs in the C-terminal half typical of all TGB1sequences and an extended N-terminal domain found in hordei-like TGB1's that do not share obvious sequence identities. TGB1 binds RNA and is thought to interact with genomic RNAs to facilitate movement. The sequence of the second TGB protein is the most conserved with BSBV, BVQ and PMTV sharing 63–75 % sequence identity and 49 % identity with that of BBNV; there is little

sequence identity among the TGB3 sequences. Analysis shows that TGB2 and TGB3 proteins contain two hydrophobic regions (predicted transmembrane domains) separated by a hydrophilic domain and these proteins are associated with intracellular membranes in infected plants. The CP and RT proteins are encoded on RNA2 of BSBV, BVQ, and BBNV (RNA3 in PMTV). The RNA2 of pomoviruses (RNA3 in PMTV) is of variable size, from 2315 to 3134 nt. Analysis of naturally occurring and glasshouse-propagated isolates revealed that deletions of c. 500–1000 nucleotides occur in both field and laboratory isolates and that they occur predominantly in the RT domain which is correlated with loss of transmission by the natural vectors (Torrance, 2008). The RT domain of BVQ is shorter than the others but recently it was evidenced that the common RT of 76 kDa is longer than the first reported reference of BVQ RNA2 (35 kDa) and has three nt additions in its C-terminal part (5, 285 and 1) then it was hypothesized that multiple inoculation cycles on *Chenopodium quinoa* were responsible for these three deletions in the C-terminal part of the previously described BVQ RNA-2. Two putative transmembrane domains, TM1 and TM2, were predicted in the consensus amino acid sequence of the ten BVQ strains, and the putative BVQ TM2 was aligned with that of *Potato mop-top virus* (Crutzen *et al.*, 2009a). The RT coding sequence of BVQ is followed by two additional ORFs for proteins of predicted mass of 9 and 18 kDa. Amino acid sequence comparisons revealed conserved motifs between these two proteins and domains in the C-terminal portions of BSBV and PMTV RT proteins which hypothesized that they might be have arisen by degeneration of a larger ORF. Variable base composition is found in natural isolates of BSBV in the sequence at the 30 end of RNA3 between the stop codon of the third TGB and the terminal tRNA-like structure (Torrance, 2008).

I.2.3.2 Virus–host interactions and movement

Cytoplasmic inclusions of enlarged endoplasmic reticulum p0050 (ER) and the accumulation of distorted membranes and small virion bundles can be seen by electron microscope examination of thin sections of BSBV- and BVQ-infected leave. In PMTV-infected potato leaves, abnormal chloroplasts with cytoplasmic invaginations were seen in thin sections as well as tubular structures in the cytoplasm associated with the ER and tonoplast and in the vacuole. PMTV does not require CP for movement and it is thought that TGB1 interacts with viral RNA forming a movement competent ribonucleoprotein (RNP) complex. Studies of transiently expressed PMTV TGB proteins fused to marker proteins such as green fluorescent protein or

monomeric red fluorescent protein in epidermal cells of *N. benthamiana* have helped to elucidate events in intracellular trafficking and indicate that PMTV interacts with the cellular membrane recycling system. PMTV TGB2 and TGB3 were shown to co-localize on the ER and in small motile granules that utilize the actin–ER network to reach the cell periphery and plasmodesmata (PD) and TGB3 contains a putative tyrosine sorting signal (Y-Q-D-L-N), mutation of which inhibits PD localization. TGB2 and TGB3 act together to transport GFP-TGB1 to the PD for movement into neighboring cells and TGB2 and TGB3 have the capacity to gate the PD pore. TGB2 co-localizes in endocytic vesicles with the Rab 5 ortholog Ara 7 (AtRabF2b) that marks the early endosomal compartment. Also, protein-interaction analysis revealed that recombinant TGB2 interacted with a tobacco protein belonging to the highly conserved RME-8 (receptor mediated endocytosis-8) family of J-domain chaperones, essential for endocytic trafficking (Torrance, 2008).

I.2.3.3 Host range of pomoviruses

Pomoviruses have a limited host range and are transmitted in soil by zoosporic plasmodiophorid vectors that have been classified as protists. Viruses that are transmitted by plasmodiophorid vectors include pomo-, peclu-, furo-, beny-, and bymoviruses and they are thought to be carried within the zoospores. The vector life cycle includes production of environmentally resistant thick-walled resting spores and viruliferous resting spores can survive in soil for many years. BVQ and BSBV are often found associated with BNYVV; BVQ and BSBV are reported only from sugar-beet-growing areas worldwide.
The viruses are thought to be transmitted in soil by *Polymyxa betae*. BVQ is mechanically transmissible only to *C. quinoa* and BSBV to members of the *Chenopodiaceae* (Torrance, 2008).

I.2.3.4 *Beet soil-borne virus* and *Beet virus Q* distribution and its role in rhizomania disease

The *Beet soil-borne virus* (BSBV) and the *Beet virus Q* (BVQ) are detected in sugar beet. BSBV is composed of three positive single stranded RNA molecules (Koenig *et al.*, 1996; Koenig *et al.*, 1997a; Koenig & Loss, 1997) (Fig. 4, 5). First detected in UK (Henry *et al.*, 1986), BSBV was also detected in Belgium, Germany, Sweden, Iran and China (Verhoyen *et al.*, 1987; Lesemann *et al.*, 1989; Lindsten,

1989; Meunier *et al.*, 2000; Farzadfar *et al.*, 2007; Wang *et al.*, 2008). Two serotypes of the virus, "Alhum" and "Wierthe" have been described. However the "Wierthe" serotype has been reclassified as *Beet virus Q* based on molecular data (Koenig *et al.*, 1998). Little is known about BVQ. However, the general genome organization of this virus closely resembles that of BSBV. Currently, it is unknown if BSBV and BVQ play a role in the rhizomania disease. Different studies have raised questions about their effects on sugar beet culture (Lindsten, 1989; Hutchinson *et al.*, 1992; Prillwitz & Schlösser, 1992; Kaufmann *et al.*, 1993; Rush & Heidel, 1995; Koenig *et al.*, 2000). Observation of reduction of BNYVV concentration and symptoms in sugar beets pre-infected by BSBV has been reported (Meunier, 2005).

I.2.4. The vector *Polymyxa betae*

BNYVV, BSBMV, BSBV and BVQ are transmitted to the host plant by the biotrophic plasmodiophorid *Polymyxa betae*. First described by Keskin (1964), it is an obligate parasite (intracellular) of sugar beet. The long persistence of *P. betae* resting spores in the soil allows, when sheltering the virus, to infect sugar beet more than ten years after the last crop. Therefore, it is impossible to get rid of it even with long rotation. *Polymyxa betae* is able to survive in the soil by mean of thick walled resting spores, named sporosori, released during root decay (Keskin, 1964). The virus is carried internally by *Polymyxa betae* resting spores in the soil, although the relationship between inoculum potential and the actual inoculum density of viruliferous *Polymyxa betae* population is currently unknown (McGrann *et al.*, 2009).

I.2.4.1 Vector taxonomy

Polymyxa betae, *Polymyxa graminis*, and *Spongospora subterranea* do not cause so important damages to host plants. A proper taxonomy of these soil-borne pathogens was not properly done due to the complicated difficulties that exist for working with this obligate parasite. Molecular characterizations proved that the best place for this obligate parasite is within the *Protozoa* (Braselton, 1995; Ward *et al.*, 2004). They characterized by: obligate intracellular parasitism, cruciform nuclear division, zoospores with two, anterior, unequal whiplash flagella, multinucleate plasmodia, and environmentally resistant, long-living resting spores (cysts) that are often clustered together to form a sporosorus (cystosorus). Development of zoospores and long-living sporosori are also typical for plasmodiophorids

(Adams, 1990; Barr, 1992; Braselton, 1995; Littlefield & Whallon, 1999; Sherwood & Rush, 1999; Pferdmenges, 2007).

I.2.4.2 Life cycle of *Polymyxa betae*

In favorable conditions, the resting spores will evolve into zoospores (Fig. 6). This bi-flagellated zoospore is able to swim in humid soil short distances to find a suitable host like epidermal cells of rootlets of sugar beet. After a host was found, the zoospores attach to the surface of the roots, encyst, and make a dagger-like body which will be used to penetrate the host cell wall and then injecting its content into the host cell forming a plasmodium. This plasmodium is a cytoplasm mass and nucleuses surrounded by a membrane (Keskin, 1964; McGrann *et al.*, 2009).

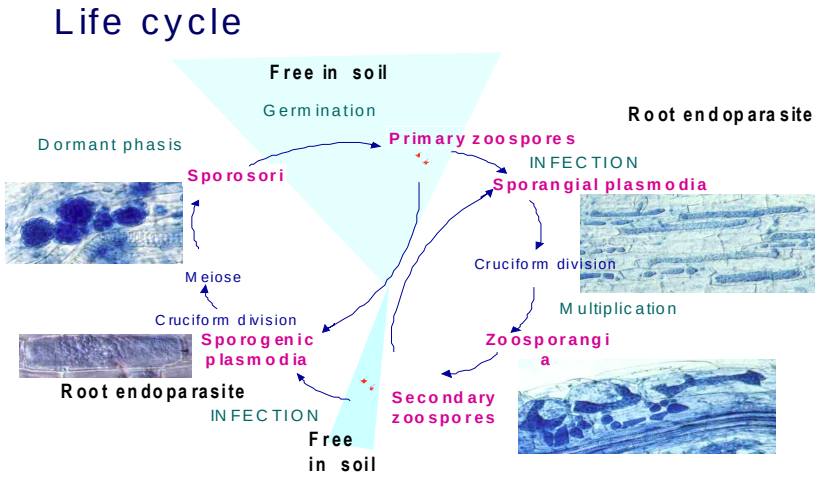


Fig. 6 Life cycle of *Polymyxa* (cycle diagram kindly provided by Anne Legrève).

This plasmodium form will give plamodiphores which produced secondary zoospores able to re-infect other cells or will enter into resting spores under certain conditions. Not only parameters such as soil humidity and temperature (Goffart & Maraite, 1992; Prillwitz & Schlösser, 1992; Tuitert, 1993; Tuitert & Hofmeester, 1994; Pferdmenges, 2007) but also basic pH and high concentrations of calcium and magnesium in the soil (Legrève & Maraite, 1992; Krabovic & Kralova, 1996), influence the infection of the plant by *Polymyxa betae*. During its cycle, *P. betae* will acquire soil-borne viruses and is said to be viruliferous. The percentage of viruliferous *P.*

betae in the soil could range from 5 to 15% (Ciafardini, 1991). The cycle of *Polymyxa betae* is completed in 7 to 10 days in normal conditions (Cooper & Asher, 1988). The precise mechanism of acquiring the virus is unknown however it is evident that viral regions are involved in the process (Dessens & Meyer, 1996; Tamada *et al.*, 1996b; Diao *et al.*, 1999; Koenig, 2000; Adams *et al.*, 2001), presumably through the interaction of membrane of the thallus and transmemberane motifs of p75 readthrough protein of virus and may accumulate in the plasmodia. Virus-like particles are observed within numerous vacuoles in young immature plasmodia or zoospores (Tamada, 1975; Abe & Tamada, 1986; Rysanek *et al.*, 1992). *Polymyxa betae* will then be able to transmit it to the plant. It is unknown if the virus is able to replicate in the vector (Adams, 1991; Campbell, 1996). Nevertheless Lubicz *et al.* (2007) reported that *P. betae* might be a host as well as a vector for association of BNYVV due to presence of replication, assembly and movement proteins of BNYVV with sporangial and sporogenic stages of *P. betae* which in turn indicates that BNYVV resides inside its vector during more than one life cycle stage and playing a role as a host for BNYVV. This needs to further investigated to determine whether *P. betae* is actually a host for BNYVV or simply a vehicle for virus transmission (Lubicz *et al.*, 2007; McGrann *et al.*, 2009).

Differences between isolates of *P. betae* have been pointed out in terms of vectoring BNYVV but also as causing damages to the sugar beet (Gerik & Duffus, 1988). Also, recent studies emphasize the existence of special forms within *Polymyxa* species according to the host range, aggressiveness, vectoring ability and environmental requirements (Gerik & Duffus, 1988; Barr & Asher, 1992; Legrève *et al.*, 2002).

P. betae forms very persistent cytosores. These were transmitted to infected soil, and both cytosores and BNYVV could persist a very adversing condition and even pass the digestive system of the sheeps (Heijbroek, 1988).

I.3. Soil-borne sugar beet virus vectored by *Olpidium brassicae*

I.3.1. *Beet black scorch virus*

The *Beet black scorch virus* is a soil-borne virus vectored by *O. brassicae* to host plants. It is a *Necrovirus*.

I.3.1.1 *Necrovirus* genome organization

The genus *Necrovirus* encompass several virus species like *Tobacco necrosis virus* A, TNV D, *Olive latent virus* and *Leek white stripe virus, Olive mild mosaic virus* as well as the *Beet black scorch virus.* These icosaedric viruses have a short compact genome of about 3.8 kb in size with only four ORFs; a fifth potential smaller ORF was predicted within the 3'-leader of the type species TNV-A. The genome organization and expression strategy of necroviruses are similar to the genus *Carmovirus.* necroviruses have a small CP that forms smooth virions and are phylogenetically related to the *Sobemovirus* CP. This feature distinguishes the necroviruses from carmoviruses, which have a larger CP with protruding domain (Fauquet *et al.*, 2005).
Virions are approximately 28 nm in diameter and exhibit a T=3 icosahedral symmetry. The shell has a smooth appearance (Fig. 7).

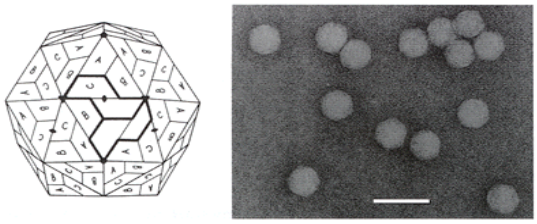


Fig. 7 (**Left**) Diagram of (T = 3) *Tobacco necrosis virus A* (TNV-A) virion. (**Right**) Negative contrast electron micrograph of TNV-A virions.The bar represents 50 nm (Fauquet *et al.*, 2005).

Virions contain one molecule of infectious linear positive sense ssRNA. The type species RNA is 3684 nt. The 5'-end of the RNA does not have a covalently linked virion protein and is uncapped, possessing a ppA terminus. The RNA does not contain a 3'-terminal poly(A) tract. The virion packages exclusively the genomic RNA. Three virus specific dsRNA corresponds to that of the genomic RNA. The second largest 1.6 kbp and the smallest 1.3 kbp dsRNA correspond to sgRNA1 and 2 respectively. Infectious RNA transcripts have been synthesized from a full-length cDNA clone of the *Olive latent virus 1* (OLV-1), TNV-D and BBSV genomes (Weiland *et al.*, 2007). The capsid is composed of 180 copies of single CP species (32 capsomeres per nucleocapsid). This protein has 268-275 amino acids and a size of 29-30 kDa.

The genomic RNA contains five or six ORFs. However ORF1 is capable of encoding a 22-23 kDa polypeptide. Readthrough of the ORF1 amber termination codon allows translation to continue into ORF1-RT for the expression of an 82 kDa polypeptide. The 82 kDa protein is predicted to be the RdRp (holding a GDD motif). ORF2 can encode 7-8 kDa polypeptide that also may be implicated in cell-to-cell movement. ORF3 encodes a 6-7 kDa polypeptide that also may be involved in movement. ORF4 encodes the 29-30 kDa CP. ORF5, present only in the TNV-A, encodes a 6-7 kDa protein. Two sgRNAs of 1.6 and 1.3 kb are synthesized in infected cells. The smaller sgRNA is the translational template for CP and the larger is the translational template for the ORF2 and possibly ORF3 products. The function of the ORF5 product is not known. The 8K protein encoded by the OLV-1 genome was detected in close proximity to plasmodesmata or within plasmodesmata channels and accumulates in the cytoplasm of infected cells as bundles of thin filaments.

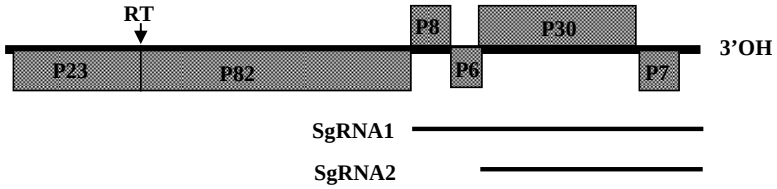


Fig. 8 Genome organization and replication strategy of *Tobacco necrosis virus A* (TNV-A). Boxes represent known and predicted ORFs with the sizes of the respective proteins (or readthrough products) indicated within. Shaded ORFs indicate polymerase proteins that have a high degree of sequence conservation within the *Tombusviridae*. Right-hatched box identifies the capsid protein (CP) that is highly conserved among other genera within the *Tombusviridae* that lack a protruding domain. P8 identifies one of the two proteins involved in cell-to-cell movement that exhibits sequence conservation with a like protein in the genera *Avenavirus*, *Carmovirus*, *Machlomovirus*, and *Panicovirus*. Lines underneath the gene map depict the two sgRNAs that are synthesized in infected cells; these allow for the expression of ORFs 2 and 3 and ORF4 from sgRNA-1 and 2, respectively. RT = amber termination codon that can be readthrough.

Necroviruses have wide host ranges that include monocotyledonous and dicotyledonous plants. In nature, infectious viruses are typically restricted to roots. Experimental inoculations usually cause necrotic lesions on the inoculated leaves, but rarely result in systemic infection. Virions are readily transmitted by mechanical inoculation. Member viruses are soil-borne. Some (TNV-A, TNV-D, *Beet black scorch virus*; BBSV) are naturally transmitted by *Olpidium brassicae*, while

others (OLV-1) are transmitted through the soil without the apparent intervention of a vector (Fauquet *et al.*, 2005).
TNV-A and TNA-D are ubiquitous, OLV-1 was reported from several Mediterranean countries, *Leek white stripe virus* (LWSV) from France, and BBSV from China (Liu & Xian, 1995; Zhang, 1996; Li *et al.*, 2008a), USA (Weiland *et al.*, 2007), Iran (Koenig & Valizadeh, 2008) and several European countries (Gonzàles-Vàzquez, 2009).

I.3.1.2 *Beet black scorch virus* (BBSV)

The *Beet black scorch virus* (BBSV) was first identified in Inner Mongolia, China, in the late 1980s (Liu & Xian, 1995; Zhang, 1996; Li *et al.*, 2008a). BBSV is a *Necrovirus* of *Tombusviridae* family (Russo *et al.*, 1981; White & Nagy, 2004). Recently it was reported from USA (Weiland *et al.*, 2007), Iran (Koenig & Valizadeh, 2008) and several European countries (Gonzàles-Vàzquez, 2009) (Fig. 9).

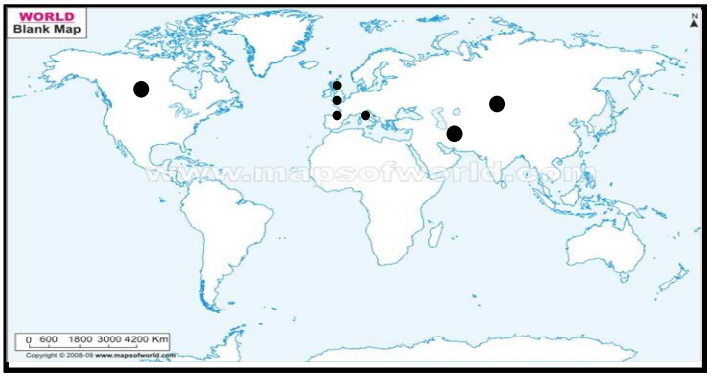


Fig. 9 Map of the BBSV geographical distribution in the world

The virus elicits severe, systemic disease symptoms described as black scorched leaves in China. The BBSV isolates reported from US did not show such a black scorching in leaves of sugar beet (*Beta vulgaris* L.). This virus apparently exacerbates symptoms of Rhizomania disease of sugar beet like rootlet proliferation (Weiland *et al.*, 2007).
After mechanical inoculation of BBSV in the glasshouse, necrotic local lesions appear on the leaves of *Chenopodium spp*., three days post inoculations. Virus can be detected in each of these hosts by RT-PCR and by retro-inoculation to *Chenopodium quinoa*.

I.3.1.3 Genome organization of *Beet black scorch virus*

The virus comprises an icosahedral particle of 28 nm that encapsidates a positive-sense, single-stranded (ss) genomic (g) RNA of 3644 nucleotides (Fig. 10). BBSV shares the highest sequence identity (61%) with *Tobacco necrosis virus* D (TNV-D) (Coutts *et al.*, 1991). Complete sequences of one Chinese (BBSV-N) and one US (BBSV-CO) BBSV isolates showed they have seven and eight open reading frames (ORFs) respectively.

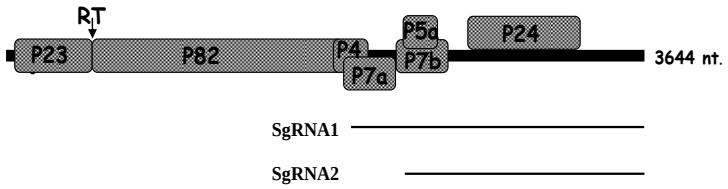


Fig. 10 BBSV genome organization

The *P23* and *P82* ORFs are thought to encode the viral RNA polymerase, as the p82 protein contains a GDD motif that is conserved in RNA-dependent RNA polymerases (Argos, 1988). The P82 ORF, residing between nucleotides 36 and 2210, is probably expressed directly from the gRNA via translational readthrough of an amber stop codon.
The small ORF of *P5'*, *P7a* and *P7b* ORFs which are located in the central region of the BBSV RNA genome are involved in cell to cell movement of the virus in host plants and each of these genes was required for localized movement, accumulation of viral RNAs and formation of local lesions on the leaves of *Chenopodium amaranticolor*, as it has been evidenced that similar proteins encoded by subgenomic (sg) RNA in TNV-D (Coutts *et al.*, 1991) and TNV-DH are required for cell-to-cell movement (Drouzas, 1996; Molnar *et al.*, 1997; Yuan *et al.*, 2006).
The 39-proximal ORF (nt 2647–3345) in BBSV is predicted to encode the capsid protein (p24) due to its 24,5 kDa mass, as estimated by SDS-PAGE. The coat proteins of TNV-DH (Molnar *et al.*, 1997) and *Turnip crinkle virus* (TCV) (Cohen *et al.*, 2000) have been demonstrated to be involved in long-distance movement during systemic infection of *Nicotiana benthamiana*, but no information is yet available about this requirement for BBSV.

There was shown previously that two sgRNAs along with the viral gRNA are present in plant tissues infected with BBSV (Fig. 10), but the sgRNAs are not encapsidated in viral particles (Guo *et al.*, 2005). It presumed that the ORFs located downstream of the putative BBSV RNA polymerase subunits use these sgRNAs for their translation, as is the case for many positive strand RNA viruses (Miller & Koev, 2000). Normally, only a single RNA was associated with infections, but a small single-stranded RNA (ssRNA) that is similar to the satellite RNAs (sat-RNA) of other necroviruses (Van Regenmortel *et al.*, 2000) has been found in isolates from the Xinjiang province of China (Cai, 1993). The sat-RNA of *Beet black scorch virus* belongs to the small linear satellite RNA subgroup, which contain several subviral RNAs associated with helper viruses in the *Tombusviridae* family, including *Turnip crinkle carmovirus*, *Cymbidium ringspot tombusvirus* (CyRSV), *Tomato bushy stunt tombusvirus* (TBSV) and *Cucumber necrosis tombusvirus* (CuNV). The BBSV satellite RNA genome is similar in size to the satellite RNAs of CyRSV (~0.7 kb) and TBSV (~0.7 kb) (Guo *et al.*, 2005). This small RNA multiplied in test plants only in presence of BBSV genomic RNA (helper virus), either from the same viral isolate or from other sources of BBSV (Bo, 1996). The presence of sat-RNA resulted in more local lesions on the leaves of *Chenopodium amaranticolor* plants then did inoculation with BBSV RNA alone when the inoculum was highly diluted this is like many sat-RNAs of plant viruses such as *Turnip crinkle virus* (TCV) (Carpenter *et al.*, 1991) and *Cucumber mosaic virus* (CMV) (Kuroda *et al.*, 1997).

I.3.1.4 Satellite RNAs and Satellite viruses

The classical definition of a satellite is a linear or circular RNA that requires a helper virus to supply proteins for replication but shares little sequence relatedness with the helper virus and is not required for the accumulation of the helper virus. Satellite that encode their own coat protein, four of which have been described, were labeled as satellite viruses to distinguish them from satellite RNAs (satRNAs), which are encapsidated in particles assembled from helper virus CP. SatRNAs are roughly divided into two classes: large satRNAs that generally encode a single nonstructural protein and smaller satRNAs with no functional coding capacity (Simon *et al.*, 2004). The satellite viruses and RNAs can be distinguished from defective interfering (DI) RNAs by the absence of significant sequence homology with their helper viruses (Roossinck *et al.*, 1992).

I.3.1.5 Gene silencing mechanism

RNA silencing collectively refers to the suppression of gene expression through nucleotides sequence-specific interactions that are mediated by RNA (Moissiard & Voinnet, 2004).RNA silencing also known as RNA interference (RNAi) in animals, is a universal system in multicellular organisms in which RNA expression is specifically downregulated at the post-transcriptional level by a complex process involving small RNAs. Among these, the so-called small interfering RNAs (siRNA) are 5'-phosphorylated molecules of 21-24 nucleotides (nt) generated by an RNAase III-type Dicer enzyme from a double-stranded (ds) RNA. One strand of the siRNA can be incorporated into an RNA-induced silencing complex (RISC) to serve as guide RNA to direct degradation of RNA species containing a complementary sequence. The core component the RISC complex is an ARGONAOUTE (AGO) protein, which contains the endonucleolytic cleavage activity. An important second class of small RNAs are the microRNAs (miRNAs) produced by Dicer from imperfect hairpin in noncoding transcripts of cellular origin. When incorporated into RISC they either direct cleavage (most commonly in plants) of transcripts bearing sequence homology of interfere with their translation. In plants and invertebrates, virus infection is typically accompanied by the appearance of double-stranded-RNAs and this can trigger a potent RNA silencing response aiming at degrading the foreign RNA. As a counter-strategy viruses encode silencing suppressor proteins (SSPs) that can block this host defense response. The molecular basis for suppressor activity has only been elucidated for a few of those proteins. For instance, the p19 protein encoded by *Tomato bushy stunt virus* binds directly to siRNAs so as to impede their loading on RISC, their spread or their amplification by cellular RNA dependant RNA polymerase. Other SSPs, including the *Closterovius* p21and HC-Pro of *Potyvirus* can also bind siRNA. This has led to the hypothesis that binding of siRNA may represent a widespread counter-strategy. Until recently, the only SSP that was known to function by interacting with a protein component of the silencing pathway was the 2b protein of *Cucumber mosaic virus*. By physically binding to AGO1, 2b provokes inhibition of the cleavage activity of the RISC machinery. More recent evidence now reveals that another SSP, the *Polerovirus* p0 protein, has an even more dramatic impact on the fate of AGO1, possibly by hijacking component of the cellular ubiquitination pathway to promote degradation of AGO1 (Bortolamiol *et al.*, 2008; Sun *et al.*, 2009).

I.3.1.6 Host-range of *Beet black scorch virus* by mechanical inoculations

BBSV can cause necrotic and chlorotic local lesions after mechanical inoculation on *Spinacia oleracea* (*Chenopodiaceae*), and just necrotic local lesions on *Tetragonia expansa* (*Aijoaceae*), *Beta macrocarpa* (*Chenopodiaceae*), *Beta vulgaris* (*Chenopodiaceae*), *Chenopodium amaranticolor* (*Chenopodiaceae*), *C. capitatum*, *C. murale*, *C. quinoa* and also it can cause symptomatic systemic infections on *Nicotiana benthamiana* (*Solanaceae*). Up to now in nature it found in sugar beet plants.

I.3.2. The Vector *Olpidium brassicae*

Olpidium is an obligate intracellular parasite of roots, produces unwalled, motile zoospores as part of the life cycle and has environmentally resistant resting spores. Its zoospore has a single, posteriorly placed whiplash flagellum and display a characteristic "jerky"swimming pattern. The zoospores are spherical and only approximately 3.3 x 5.6 μm (Rochon *et al.*, 2004).
Most of the current understanding of the *Olpidium* life cycle comes from early studies described by Temmink. The initial stage of zoospore encystment likely involves withdrawal of the flagellum (composed of axoneme plus axonemal sheath) into zoospore body; however, the possibility that the flagellum wraps around the developing cyst has not been ruled out. Electron micrographs of newly encysted zoospores often show a "whorl of membranes" in association with the plasmalemma inside the developing cyst. The presence of this feature suggests the axonemal sheath of the flagellum may be drawn into the body of the zoospore along with the axoneme and that the axonemal sheath breaks during this process resulting in the observed whorl of membranes. The penetration of the host cell involves the production of a variable-size papillum produced by the host cell in response to *Olpidium* infection. In addition, a hole appears in the cyst at the junction of papillum. The cyst protoplast then appears to move into the host cell cytoplasm through a hole in the host cell plasma membrane. The cyst cytoplasm, in conjunction with a newly formed plasmalemma, is released into the host cell, leaving behind the original plasmalemma, the tonoplast, and cyst wall. The thallus enlarges over the next one to two days, still separated from the host cytoplasm by only a thin membrane. Thalli that will develop into zoosporangi become multi-nucleate and develop a thick wall around the thallus outside of the plasmalemma. Cleavage vesicles form about one day later and then they fuse to delineate the individual zoospores

of the zoosporangium. Concomitant with the formation of the mature zoosporangium is the formation of an exit tube in the zoosporangium from which many mature zoospores are released following contact with water. The thallus may also develop into resting spores, but in this case, it does not become multinucleate. Resting spore formation is not believed to involve a sexual stage (Rochon *et al.*, 2004; Astier *et al.*, 2007).

The BBSV has been shown to be transmitted efficiently through the soil in a non-persistent manner by zoospores of *Olpidium brassicae* (*Chytridales*) (Jiang, 1999). Typical *in vitro* virus transmission is demonstrated between *Olpidium brassicae* and *Tobacco necrosis virus* (TNV), where virus transmission is much more independent from the vector. Once, *O. brassicae* transmits TNV virions, which are only absorbed to the surface of fungal membrane, the virus propagate in the host-cell and further spread is independent from the vector. If the host cell dies, fungal independent TNV virions get released into the soil (Campbell, 1996). Viruses transmitted by *Olpidium brassicae*, such as TNV, STNV and CuNV, are carried on the surface of the spores. Zoospores carrying virus probably survive for only a few hours. However, virus that is free in the soil may be picked up and transmitted by newly released zoospores. If the soil is moist, *Olpidium* zoospores can recover TNV from soil for at least 11 weeks (Hull, 2002). Viruses transmitted by *Olpidium* do not survive in air-dried soil. In general, viruses of this type have wide host ranges and probably survive in the soil by frequent transmission to successive host. Drainage water and movement of soil and root fragments are probably important in the spread of these viruses from one site to another (Hull, 2002).

A recent survey on different isolates of *Olpidium brassicae* from distantly separated areas of the world showed that they could be separated into two distinct genetic groups of brassica-infecting isolates, two distinct groups of lettuce-infecting isolates, one of which contained a carrot-infecting isolate and a distinct group comprising a cucumber-infecting isolate and a melon-infecting isolate (Hartwright *et al.*, 2009).

Chapter II. Objectives

II.1. General objective of the thesis

Sugar beet in Iran is a crop under high disease pressure. This work started from fields showing strong root proliferation symptoms and foliar symptoms, with the question of the reasons for such major impact on the beets. To unravel the problem, convergent research strategies combining epidemiological and spatial diversity approaches together with a systematic analysis of the viral genome diversity was chosen. The general objective of this work was to identify the different agents involved in the soil-borne viruses BNYVV, BSBV, BVQ and BBSV occurring in Iran and to better understand their contribution to the rhizomania syndrome.
Soil-borne viruses from genus *Benyvirus*, *Pomovirus* and *Necrovirus* have been searched for by RT-PCR and sequencing procedure.

II.2. Scientific strategy

II.2.1. Mapping soil-borne viruses

All provinces of Iran where sugar beet is cultivated have been explored for the presence of soil-borne viral diseases. By visual screening and using a smart sampling strategy, more than 300 samples were gathered from different parts of the country.
Based on soil bioassays under control conditions, the different viruses present were detected and studied.

II.2.2. Genetic diversity of BNYVV

To evaluate the impact of BNYVV, the major soil-borne virus known on sugar beet, the presence of P type isolate as well as the association with RNA-5 was evaluated. Furthermore, the diversity of the p25 located within the RNA-3 was studied to understand whether the presence of special isolates could explain the virulence observed in the fields. The different partial sequences of BNYVV were compared to the one from other parts of the world especially from Belgium and France.

II.2.3. Presence of BSBV and BVQ

For this purposes we used multiplex RT-PCR developed by Meunier *et al.* (2003). In sugar beet fields infected by BNYVV, pomoviruses are commonly detected in other parts of the world. Even if their role in the etiology of the disease remains unclear, and since their presence

was not fully assessed in Iran, the presence of the BSBV and the BVQ was evaluated by RT-PCR. This part of the work contributed also to the construction of full length clones for *Beet soil-borne virus* which will help to understand the exact role of pomoviruses in the disease.

II.2.4. Evaluating the presence of new soil-borne viruses in Iranian samples

Other soil-borne virus have also been reported on sugar beet, which were not reported in Iran at the beginning of this work, like the *Beet soil-borne mosaic virus* and the *Beet black scorch virus*. We also evaluated their presence in Iranian sugar beet fields.

II.2.5. Genetic diversity of BBSV

Following the discovery of BBSV, the genetic diversity of the virus was studied by comparing the sequences of six full length clones. A special attention was paid to the 3' untranslated region, which is of uttermost importance for the replication and translation process of the viral genome, in relation with the presence of the virus satellite.
Finally, the pathogenicity of the different studied isolates was evaluated on sugar beet as well as on *Beta macrocarpa*, *Spinacia oleracea*, *Nicotiana benthamiana* and *Chenopodium quinoa*, with the view of understanding the pathogenicity determinants (Some 'guinea pigs' widely used as test plants for infectivity assay and detection which includes the *Chenopodiaceae Chenopodium amaranticolor*, *Chenopodium quinoa*, the *Leguminosae Phaseolus vulgaris*, *Vicia faba* and *Vigna unguiculata*, the *Solanaceae Nicotiana benthamiana*, *Nicotiana clevelandii*, *Nicotiana glutinosa*, *Nicotiana occidentalis*, *Nicotiana tabacum* and *Petunia hybrida*, and cucurbitaceous *Cucumis sativus*. These plant species are also useful as **experimental hosts** for virus propagation and can easily and rapidly be grown in large numbers) (Bos, 1999).

Chapter III.

Iranian *Beet necrotic yellow vein virus* (BNYVV): Pronounced Diversity of the P25 Coding Region in A type BNYVV and Identification of P type BNYVV Lacking a Fifth RNA Species

Arch Virol (2009) 154:501–506
DOI 10.1007/s00705-009-0322-z

BRIEF REPORT

Iranian beet necrotic yellow vein virus (BNYVV): pronounced diversity of the p25 coding region in A-type BNYVV and identification of P-type BNYVV lacking a fifth RNA species

M. Mehrvar · J. Valizadeh · R. Koenig · C. G. Bragard

Received: 12 October 2008 / Accepted: 14 January 2009 / Published online: 15 February 2009

Abstract Beet necrotic yellow vein virus (BNYVV) was detected in 288 of the 392 samples collected in Iran. A-type BNYVV was detected most frequently. The p25 coding region on BNYVV RNA-3 was amplified by RT-PCR and sequenced. Nine different variants of the highly variable amino acid tetrad at positions 67–70 of p25 were identified, i.e. ACHG, AHHG, AYHG, ALHG, AFHR, AFHG, AHYG, VLHG and VHHG. These are more different tetrad variants than have been reported from any other country. The first three variants were found most commonly. In 23 out of the 288 BNYVV-positive samples, we detected P-type BNYVV that had previously been identified only in France, Kazakhstan and recently in the UK. Surprisingly, none of these samples contained the fifth RNA species usually associated with P-type BNYVV in other countries. As in other BNYVV P-type sources, the p25 amino acid tetrad in positions 67–70 of the Iranian P-type consists of SYHG.

M. Mehrvar · C. G. Bragard (✉)
Université Catholique de Louvain,
Unité de Phytopathologie, Louvain-la-Neuve, Belgium
e-mail: claude.bragard@uclouvain.be

M. Mehrvar
Department of Plant Protection, Faculty of Agriculture,
Ferdowsi University of Mashhad, Mashhad, Iran

J. Valizadeh
Department of Biology, Faculty of Science,
University of Sistan and Baluchestan, Zahedan, Iran

R. Koenig
c/o Julius-Kühn-Institut (JKI), Bundesforschungsinstitut
für Kulturpflanzen, Institut für Epidemiologie und
Pathogendiagnostik, Braunschweig, Germany

Rhizomania is a major disease of sugar beet with worldwide distribution [2, 32]. In Iran, it was reported from the Fars province in 1996 and is now found in nearly all sugar-beet-growing areas of the country [11, 35]. Beet necrotic yellow vein virus (BNYVV) is responsible for the disease.

Molecular analysis of the four RNA species always present in natural BNYVV infections has revealed the existence of three major strain groups named A, B and P-types [15, 17, 20, 22, 23, 31, 42]. These three types were found to be highly conserved in Europe: recently determined sequences are practically identical to those determined more than two decades ago by Bouzoubaa et al. [3–5]. BNYVV sources from East Asian countries (Japan, China) are similar to the European A- and B-types, but not completely identical to them [17, 28, 30, 42].

The RNA-3-encoded p25 enables the systemic movement of the virus in sugar beet and is mainly responsible for symptom expression [12, 14, 36] and root proliferation [38]. Its ability to shuttle between the nucleus and the cytoplasm is necessary for symptom expression on leaves of *Chenopodium quinoa* [41]. The amino acid tetrad in positions 67–70 of p25 has been found to be highly variable in BNYVV sources collected at many different places in the world [18, 25, 29, 31, 34, 39, 42]. Chiba et al. [6, 7] found that the resistance response on leaves of selected lines of *Beta maritima* and the sugar beet variety Rizor depended on the composition of this tetrad. p25 was thus identified as an avirulence factor. Klein et al. [13] found that sequence variations of p25 influenced its oligomerization and pathogenicity in *Tetragonia expansa*. Acosta-Leal and Rush [1] identified $V_{67}E_{135}$ in p25 as the signature for resistance-breaking variants of BNYVV from the US as opposed to the WT motif $A_{67}D_{135}$. Koenig et al. [19] found that V_{67} P25 promoted a much higher virus accumulation in the rootlets of mechanically inoculated, partially resistant

sugar beet seedlings than A_{67} P25. Other variants of the tetrad have also been associated with resistance breaking [26, 32].

The presence of a fifth RNA species in the BNYVV genome has also been associated with an increased pathogenicity of BNYVV [37]. In East Asia, BNYVV RNA5 has been found to be frequently associated with A- or B-type-like BNYVV [24, 30]. A distinct type of RNA 5 has been found to be associated with P-type BNYVV, a virus type which so far has been detected only in France, Kazakhstan and, more recently, in the UK [10, 16, 17, 42].

Sequence information is mainly available for European, American and East Asian BNYVV isolates. The present study provides a comprehensive overview of the distribution of various BNYVV types in Iran.

Sugar beets with rhizomania-like symptoms or grown as bait plants in soil samples were obtained for the Iranian locations listed in Table 1. Most of the samples came from different fields. A limited number (13 samples out of 5 locations) were collected at the same place, but always from fields larger than 25 ha. The p25 coding region on BNYVV RNA 3 and various portions of RNAs 1, 2 and 4 were amplified by means of RT-PCR or immunocapture RT-PCR as described by Meunier et al. [27] and Koenig et al. [15]. The nucleotide sequences of the PCR products were obtained by using an ABI377 sequencer (Applied Biosystems) and were analyzed by the Clustal W program of the Genetic Computer Group [8] and the Invitrogen Vector NTI Advance 10 program. Phylogenetic analysis of nucleotide sequences was performed using the MEGA4 software package [40], using the neighbor-joining method [33] and a bootstrap value of 1,000 for appreciation of confidence [9].

BNYVV was detected in 288 out of 392 sugar beet samples tested. The majority of the samples contained A-type BNYVV RNA 3, but in c. 8% of the samples, P-type BNYVV RNA-3 was identified (Table 1). No BNYVV B-type infections were found. The amino acid tetrad in position 67–70 of the RNA 3-encoded p25 in A-type BNYVV showed considerable variation. All together, nine different variants were identified. With frequencies of c. 28, 27 and 23%, respectively, the ACHG, AHHG and AYHG tetrads were found to occur most commonly (Table 1). P25 with an ACHG tetrad was detected mainly in Khorasan Razavi and to a lesser extent in some other provinces (Table 1). P25 with an AHHG tetrad has a countrywide distribution in most of the Iranian sugar-beet-growing areas. P25 with an AYHG tetrad was found mainly in the Fars and the Kermanshah provinces and to a lesser extent in several other provinces. P25 with ALHG, AFHR or AFHG tetrads were detected with a lower frequency and with an uneven distribution within the country (Fig. 1, Table 1). P25 with an AFHG tetrad was detected mostly in mixed infections with AYHG, ALHG, and ACHG. P25 with an AFHR tetrad was found only in the Azarbeijan Gharbi province in northwestern Iran. P25 with an AHYG tetrad was found only once in a sample from Khorasan Razavi (Jovein). VLHG (Hamadan) and

Table 1 Number of BNYVV-positive samples that were collected in various Iranian provinces and contained different amino acid tetrads at positions 67–70 of p25

Province	A-type									P-type	Total no. of samples tested
	AHHG	ACHG	AYHG	ALHG	AFHR	AFHG	AHYG	VLHG	VHHG	SYHG	
Khorasan Razavi (NE)	57 (4)	67 (5)	4 (1)	11 (1)		2 (1)	1		1 (1)	8 (2)	143
Khorasan Shomaali (NE)	12									1	13
Semnan (NE)	2										2
Ghazvine (NW)	2 (1)		7	1 (1)		1 (1)					9
Zanjan (NW)	1										1
Ardabil (NW)		2 (1)	1 (1)			1 (1)				11 (1)	13
Azarbaeijan Sharghi (NW)		4	2								6
Azarbaeijan Gharbi (NW)	3		7 (1)		7					2 (1)	18
Ilam (W)	1		2	2							5
Kermanshah (W)		7	19	10						1	37
Hamadan (W)	4	6	4	2 (1)				1 (1)			16
Fars (S)			20 (1)			1 (1)					20
Kerman (S)			5								5
Total no. of sequences with the respective tetrad variant	82	86	71	26	7	5	1	1	1	23	288 303

Mixtures of two different tetrads were found in 11 samples and mixtures of three tetrads were found in two samples. When additional sequences were found in a sample, their number and composition is given in parentheses; *NE* northeast, *NW* northwest, *S* south, *C* central

Fig. 1 Evolutionary relationships of the P25-encoding regions of BNYVV RNA-3 from various parts of the world, computed by MEGA4 software [40], using the neighbor-joining option [9] and compared to the amino acid polymorphism. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The branch lengths are in the same units as those of the evolutionary distances. The *letters* preceding the *parentheses* indicate the country of origin (*A* Austria, *C* China, *F* France, *Ir* Iran, *It* Italy, *J* Japan, *K* Kazakhstan, *N* Netherlands, *Sp* Spain, *UK* United Kingdom, *USA* United States of America. The *letters* in the *parentheses* refer to the composition of the amino acid tetrad; they are followed by the GenBank accession numbers. Sequences obtained in this work are boxed in *gray*

VHHG (Khorasan Razavi) tetrads were also detected only once in mixed infections with ALHG- and AHHG-containing p25, respectively.

The diversity observed within the p25 tetrad is the highest observed in the world for such an area. By comparison, a similar study on BNYVV diversity in which samples from six Chinese sugar-beet-growing regions were gathered revealed seven different tetrad motifs [25]. Interestingly, the proportions of the different p25 tetrads observed were completely different from those in other areas where such data have already been gathered, such as Western Europe or the USA [26, 34]. Their occurrence was possibly due to spontaneous mutations, as suggested by Acosta-Leal and Rush [1]. An explanation might be the wide use of non-resistant polygerm sugar beet cultivars in conjunction with the intense use of monogerm tolerant ones, with the possibility of raising soil infectious potential to high levels and then confronting such soils with virus resistance genes. Although it was not possible to gather all the information about the rhizomania-affected sugar beet cultivars, such data were collected for 151 positive samples. Most of the A-type samples were associated with the susceptible Iranian cultivar varieties, while the P-type was more often found on tolerant varieties. It is also interesting to note that BNYVV was first reported as A- and B-type in Iran [35], but now only the A- and P-types are found.

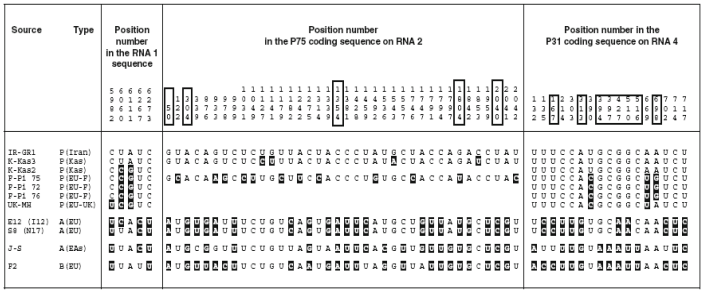


Fig. 2 Comparison of sequences of portions of RNAs 1, 2 and 4 of an Iranian P-type isolate with the corresponding sequences of P-types from other parts of the world and of other BNYVV types. To aid in the analysis, only the nucleotides in those positions are shown in which the Iranian P-type differs from other P-types or where all P-types differ from at least two other BNYVV types. The nucleotides that differ from those in the corresponding positions in the Iranian P-type are highlighted by *white letters* on a *black background. Kas* Kazakhstan, *F* France, *A(EU)* European A-types, *A(EA)* East Asian A-type, *B(EU)* European B-type. The GenBank accession numbers for partial or complete RNA sequences are FJ230959, DQ462117, DQ462116, DQ462114, DQ462113, DQ462115, DQ459316, EU330453, EU330450, D84410 and X05147 for RNAs 1 of isolates Val17-GR1, K-Kas3, K-Kas2, F-Pi 75, F-Pi 72, F-Pi 76, UK-MH, E12., S8, jap-S, F-F2, respectively; FM210683, AF197556, AF197547, EU330451, EU330452, NC003515 and X04197 for RNAs 2 of the isolates IR-GR1, K-Kas3, F-Pi 75, UK-MH, E12., S8, jap-S, F-F2, respectively, and FM210681, AF197557, AF197554, AF197548, AF197546, AF197550, AF197548, DQ440514, AF197552, AF197559, NC003517 and M36896 for RNAs 4 of isolates IR-GR1, K-Kas3, K-Kas2, F-Pi 75, F-Pi 72, F-Pi 76, UK-MH, I12., NL7, jap-S, F-F2, respectively

The amino acid compositions of the various Iranian p25 sources were also compared to those from other BNYVV sources containing p25 with the same tetrads (Fig. 1). Iranian p25 with AHHG, ACHG and AFHR tetrads had almost the same amino acid composition as the corresponding European sources. p25 containing AYHG differed from Japanese and Chinese AYHG sources in one or several amino acid positions (Fig. 1). Iranian ALHG p25 were similar to Italian and US sources but different from the Kazakh, French and Dutch sources already described. Iranian AFHG p25 was identical or similar to two European AFHG P25 sources but differed at four positions from the Japanese source. Interestingly, the four exchanges in the C-terminal half of the sequence were identical to those observed in the SYHG p25 of the P-type (Fig. 1).

The SYHG-containing p25 of the Iranian P-type had an identical amino acid composition in all 23 samples investigated. They originated from different areas in the northeast, the northwest and west of the country. This P-type was found preferentially in fields heavily cropped with sugar beet resistant varieties. The amino acid composition of this Iranian P-type p25 was very similar to that of P-type isolates from other countries (France, Kazakhstan, UK) and to that of the SYHG-containing p25 from two isolates in the USA (Fig. 1). It differed from that of other isolates, however, by a 'C' rather than an 'R' (Kazakhstan) or an 'S' (France, UK, USA) at amino acid position 30.

To check whether the other RNAs of these Iranian SYHG P25-encoding virus isolates had P-type sequences, we attempted to amplify portions of their RNAs 1, 2, 4 and 5. PCR products were readily obtained for the first three RNAs. However, with none of the 23 samples from different parts of the country were we able to obtain a PCR product with primers specific for RNA-5. RNA-5 is normally associated with P-type BNYVV in France [16, 34], Kazakhstan [17] and the UK [10, 42]. The RNA-5-free P-type has, however, been reported previously from a location in France [21], The partial sequences obtained for RNAs 1, 2 and 4 of these Iranian virus sources contained most of the nucleotide substitutions that differentiate the P-type from other BNYVV types (Fig. 2). The Iranian P-type shows a higher degree of sequence identity in all three RNAs with the P-types from Kazakhstan, especially the Kas3 isolate, than with the European P-types from France and the UK (Fig. 2). An RNA-3 coding for a SYHG tetrad has also been reported from the Imperial Valley in the USA [26, Fig. 1]. The coat proteins of these isolates, however, have typical BNYVV A-type amino acid sequences which are characterized by K_{17} T_{62} V_{102} S_{103} L_{172}, whereas P- and B-type coat proteins have R_{17} T_{62} I_{102} S_{103} L_{172} and K_{17} S_{62} V_{102} N_{103} F_{172}, respectively, [17, 23, 30, 34].

This study demonstrates the occurrence of wide variations within the RNA-3-located p25 coding region in a vast country like Iran. Looking at studies on the impact of such variations on resistance response and pathogenicity [1, 6, 7, 13, 19, 26], it stresses the need for developing efficient strategies for controlling a wide variety of different types and subtypes of BNYVV.

Acknowledgments This work was supported in part by a scholarship to M. Mehrvar from the Ministry of Science, Research and Technology, Iran, by the Walloon region (DGA, subvention D31-1146) and in part by the Arbeitsgemeinschaft industrieller Forschungsvereinigungen (AiF Grant 14163 N72, GFP BR42/04) in Germany.

References

1. Acosta-Leal R, Rush CM (2007) Mutations associated with resistance-breaking isolates of *Beet necrotic yellow vein virus* and their allelic discrimination using TaqMan technology. Phytopathology 97:325–330
2. Asher MJC (1993) Rhizomania. In: Cooke DA, Scott RK (eds) The sugar beet crop science into practice. Chapman & Hall, London, pp 311–346
3. Bouzoubaa S, Guilley H, Jonard G, Richards K, Putz C (1985) Nucleotide sequence analysis of RNA-3 and RNA-4 of *Beet necrotic yellow vein virus*, isolates f2 and g1. J Gen Virol 66:1553–1564
4. Bouzoubaa S, Ziegler V, Beck D, Guilley H, Richards K, Jonard G (1986) Nucleotide sequence of *Beet necrotic yellow vein virus* RNA-2. J Gen Virol 67:1689–1700
5. Bouzoubaa S, Quillet L, Guilley H, Jonard G, Richards K (1987) Nucleotide sequence of *Beet necrotic yellow vein virus* RNA-1. J Gen Virol 68:615–626
6. Chiba S, Miyanishi M, Tamada T (2002) Single amino acid changes in the P25 protein of *Beet necrotic yellow vein virus* determine resistance responses of *Beta vulgaris* spp. *maritima*. In: Rush CM, Merz U (eds) Proceedings of the 5th symposium of the international working group on plant viruses with fungal vectors, 22–25 July 2002. Zurich, pp 5–8
7. Chiba S, Miyanishi M, Andica IB, Kondo H, Tamada T (2008) Identification of amino acids of the *Beet necrotic yellow vein virus* p25 protein required for induction of the resistance responses in leaves of *Beta vulgaris* plants. J Gen Virol 89:1314–1323
8. Devereux J, Haeberli P, Smithies O (1984) A comprehensive set of sequence analysis programs for the VAX. Nucl Acid Res 12:387–395
9. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791
10. Harju VA, Mumford RA, Blockley A, Boonham N, Clover GRG, Weekes R, Henry CM (2002) Occurrence in the United Kingdom of *Beet necrotic yellow vein virus* isolates which contain RNA 5. Plant Pathol 51:811
11. Izadpanah K, Hashemi P, Kamran R, Pakniat M, Sahandpour A, Masumi M (1996) Widespread occurrence of rhizomania-like disease of sugar beet in Fars. Iran J Plant Pathol 32:200–206
12. Jupin I, Guilley H, Richards KE, Jonard G (1992) Two proteins encoded by *Beet necrotic yellow vein virus* RNA 3 influence symptom phenotype on leaves. EMBO J 11:479–488
13. Klein E, Link D, Schirmer A, Erhardt M, Gilmer D (2007) Sequence variation within *Beet necrotic yellow vein virus* p25 protein influences its oligomerization and isolate pathogenicity on *Tetragonia expansa*. Virus Res 126:53–61
14. Koenig R, Jarausch W, Li Y, Commandeur U, Burgermeister W, Gehrke M, Luddecke P (1991) Effect of recombinant *Beet necrotic yellow vein virus* with different RNA compositions on mechanically inoculated sugar beets. J Gen Virol 72:2243–2246
15. Koenig R, Lüddecke P, Haeberle AM (1995) Detection of *Beet necrotic yellow vein virus* strains, variants and mixed infections by examining single-strand conformation polymorphisms of immunocapture RT-PCR products. J Gen Virol 76:2051–2055
16. Koenig R, Haeberle AM, Commandeur U (1997) Detection and characterization of a distinct type of *Beet necrotic yellow vein virus* RNA 5 in a sugar beet growing area in Europe. Arch Virol 142:1499–1504
17. Koenig R, Lennefors BL (2000) Molecular analyses of European A, B and P type sources of *Beet necrotic yellow vein virus* and detection of the rare P type in Kazakhstan. Arch Virol 145:1561–1570
18. Koenig R, Kastirr U, Holtschulte B, Deml G, Varrelmann M (2008) Distribution of various types and P25 subtypes of *Beet necrotic yellow vein virus* in Germany and other European countries. Arch Virol 153:2139–2144
19. Koenig R, Loss S, Specht J, Varrelmann M, Lüddecke P, Deml G (2009) A single C/U nucleotide substitution changing alanine to valine in the *Beet necrotic yellow vein virus* P25 protein promotes increased virus accumulation in roots of mechanically inoculated, partially resistant sugar beet seedlings. J Gen Virol (in press)
20. Kruse M, Koenig R, Hoffman A, Kaufmann A, Commandeur U, Solevyev AG, Savenkov I, Burgermeister W (1994) Restriction fragment length polymorphism analysis of reverse transcription-PCR products reveals the existance of two major strain groups of *Beet necrotic yellow vein virus*. J Gen Virol 75:1835–1842
21. Lemaire O, Beuve M, Weber C, Schirmer A, Link D, Meunier A, Bragard C, Gilmer D (2003) Etiology and molecular epidemiology of a severe rhizomania disease occurring in confined locations in Europe: hypothesis for the implication of the RNA-3 and/or-5 of *Beet necrotic yellow vein virus* (BNYVV)-P pathotype. In: Proceedings of the 1st joint IIRBASSBT congress, 26 February–1 March 2003. San Antonio, pp 303–318
22. Lennefors L, Lindsten K, Koenig R (2000) First record of A and B type *Beet necrotic yellow vein virus* in sugar beets in Sweden. Eur J Plant Pathol 106:199–201
23. Lennefors BL, Savenkov EI, Mukosa SB, Valkonen JPT (2005) Sequence divergence of four soilborne sugarbeet-infecting viruses. Virus Genes 31:57–64
24. Li DW, Yu JL, Han CG, Lui T, Quin SC, Lui Y, Koenig R (1999) Detection and nucleotide sequence analysis of *Beet necrotic yellow vein virus* RNA 5 isolated from China. Chin J Biotech 15:461–465
25. Li M, Liu T, Wang B, Han C, Li D, Yu J (2008) Phylogenetic analysis of *Beet necrotic yellow vein virus* isolates from China. Virus Genes 36:429–432
26. Liu H, Lewellen RT (2007) Distribution and molecular characterization of resistance-breaking isolates of *Beet necrotic yellow vein virus* in the United States. Plant Dis 91:847–851
27. Meunier A, Schmit JF, Stas A, Kutluk N, Bragard C (2003) Multiplex RT-PCR for the simultaneous detection of *Beet necrotic yellow vein virus*, *Beet soilborne virus*, *Beet virus Q* and their vector *Polymyxa betae* KESKIN on sugar beet. Appl Environ Microbiol 69:2356–2360
28. Meunier A (2005). Typing of Belgian Beet necrotic yellow vein virus, characterization of RNA-2 and RNA-3 coding regions and relationsips with different steps of the virus cycle. PhD thesis, UCL, Louvain-la-Neuve, Belgium, 122p
29. Meunier A, Schmit JF, Bragard C (2005) Comparison of the *Beet necrotic yellow vein virus* P75 nucleotide sequences of Belgian type A and type B sources. Virus Res 108:15–22
30. Miyanishi M, Kusume T, Saito M, Tamada T (1999) Evidence for three groups of sequence variants of *Beet necrotic yellow vein* virus RNA 5. Arch Virol 144:879–892
31. Ratti C, Resca R, Lanzoni C, Rubies Autonell C (2006) Amino acid changes in the 25 protein of *Beet necrotic yellow vein virus*. In: Rush CM, Merz U (eds) Proceedings of the 6th symposium of the international working group on plant viruses with fungal vectors, 5–7 September 2005. Bologna, p 53
32. Rush CM, Liu HY, Lewellen RT, Acosta-Leal R (2006) The continuing saga of rhizomania of sugar beets in the United States. Plant Dis 90:4–15

33. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425
34. Schirmer A, Link D, Cognat V, Moury B, Beuve M, Meunier A, Bragard C, Gilmer D, Lemaire O (2005) Phylogenetic analysis of isolates of *Beet necrotic yellow vein virus* collected worldwide. J Gen Virol 86:2897–2911
35. Sohi H, Maleki M (2004) Evidence for presence of types A and B of *Beet necrotic yellow vein virus* (BNYVV) in Iran. Virus Genes 29:353–358
36. Tamada T, Shirako Y, Abe H, Saito M, Kiguchi T, Harada T (1989) Production and pathogenicity of isolates of *Beet necrotic yellow vein virus* with different numbers of RNA components. J Gen Virol 70:3399–3409
37. Tamada T, Kusume T, Uchino H, Kiguchi T, Saito M (1996) Evidence that *Beet necrotic yellow vein virus* RNA-5 is involved in symptom development of sugar beet roots. In: Sherwood JL, Rush CM (eds) Proceedings of the 3th symposium of the international working group on plant viruses with fungal vectors, Dundee, 6–7 August 1996, pp 49–52
38. Tamada T, Uchino T, Kusume T, Saito M (1999) RNA 3 deletion mutants of *Beet necrotic yellow vein virus* do not cause rhizomania disease in sugar beets. Phytopathology 89:1000–1006
39. Tamada T, Miyanishi M, Kondo H, Chiba H, Han G (2002) Pathogenicity and molecular variability of *Beet necrotic yellow vein virus* isolates from Europe, Japan, China and The United States. In: Rush CM, Merz U (eds) Proceedings of the 5th symposium of the international working group on plant viruses with fungal vectors, Zurich, 22–25 July 2002, pp 13–16
40. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599
41. Vetter G, Hily JM, Klein E, Schmidlin L, Haas M, Merkle T, Gilmer D (2004) Nucleo-cytoplasmic shuttling of the *Beet necrotic yellow vein virus* RNA-3 encoded P25 protein. J Gen Virol 85:2459–2469
42. Ward L, Koenig R, Budge G, Garrido C, McGrath C, Stubbley H, Boonham H (2007) Occurrence of two different types of RNA-5-containing *Beet necrotic yellow vein virus* in the UK. Arch Virol 152:59–73

Chapter IV.

Iranian *Beet soil-borne virus* and *Beet virus Q*

Iranian *Beet soil-borne virus* and *Beet virus Q*

Beet soil-borne virus (BSBV) and *Beet virus Q* (BVQ) are two members of the genus *Pomovirus* that are transmitted by *Polymyxa betae*. BVQ and BSBV are commonly found in fields where BNYVV the causal agent of rhizomania disease, is present (Meunier *et al.*, 2003). For the purpose of evaluating the presence, diversity and in some extend the characterization of these viruses in Iran a survey was conducted to define the situation of Iranian sugar beet pomoviruses. As these viruses are normally accompanied by BNYVV in infected sugar beet fields, we focused on their coat protein sequences for finding a possible variation within them although these viruses show less variation and then we tried to make a full-length infectious clone of Iranian BSBV which was accompanied by BNYVV in a heavily infected sugar beet field in Iran (Nyshabour, Khorasan Razavi).

Distribution and characterization of Iranian *Beet soil-borne virus*

Beet soil-borne virus was present in nearly half (one hundred and seven out of two hundred and three infected samples by BNYVV) of the Iranian samples (52.7%) mostly accompanied by BNYVV and just in 6 samples it was alone, in fifty samples (23.1 %) it was accompanied by BNYVV. In other cases the Iranian BSBV was accompanied by two viruses of BNYVV and either BVQ or BBSV. In 3.7% of the samples it was associated by BNYVV, BVQ and BBSV. Interestingly the BSBV was never found in association with BBSV which raised some questions about the possibility of interactions between sugar beet pomoviruses and BBSV or BBSV satellite in one side and on the other side the role of BNYVV in interaction between all these viruses.
In terms of symptoms pertaining to this virus there was not a specific symptom but only shared root proliferation and general yellowing of fields. Due to presence of BSBV alone in six samples of our samples we speculated that may be it could be infectious alone contrarily by BVQ which has not been found alone. Also it was distributed in most of the Iranian areas under the cultivation of sugar beet.

Coat protein sequencing of eighteen Iranian BSBV isolates

Coat protein sequences of 18 Iranian BSBV plus 7 isolates from China, Germany, France and USA showed not too much variation and that most of the genome is conserved. Totally 35 mutations out of 495 nucleotides in CP sequences were found which is less than 7 % variation in BSBV coat protein gene in nucleotide level. The major changes are in positions 99, 225, 246 and 441. In position 99, 9

Iranian isolates (36%) have residue U and 9 isolates (36%) have residue C other isolates have residue U except one US and one Chinese isolates which have residue C. In position 246, 14 Iranian isolates plus two isolates from USA and China have residue A and just 4 Iranian isolates plus isolates from China, France and Germany have residue G. In position 441, 10 isolates have residue A and 13 isolates have residue U while 2 isolates have residue G. At the rest of the CP sequences there is just 1 or 2 mutation in each isolates in different positions. But in CP amino acids sequences of 18 BSBV plus 7 isolates from China, Germany, France and USA there are just 8 changes (4.9% variation in amino acid level) in position 20, 76, 85, 110, 116, 136, 140 and 157 (Fig. 1). This showed that most of the mutations are silent and the CP sequence is conserved may be due to constraints which imposed by vector or host plant factors. Also the phylogenetic tree of CP of 18 Iranian BSBV is shown in Fig. 2. In terms of full-length sequences of different RNAs of Iranian BSBV in nt level it was 97 % identical to Polish and Chinese sources and 96 % to German source of BSBV RNA1 in nt level. In RNA2 and RNA3 it was 96 % identical to Polish, Chinese and German sources of BSBV RAN2 and RNA3 in nt level.

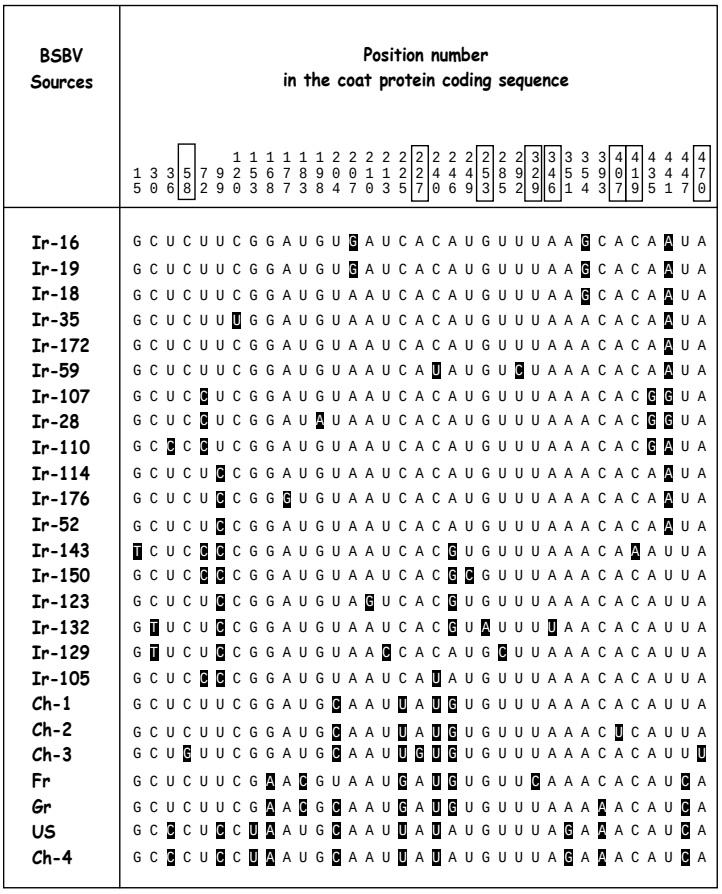


Fig. 1 Comparison of nt sequences of the coat protein of 18 Iranian BSBV by other sources of BSBV from China, France, USA and Germany. Numbers are corresponding to the BSBV coat protein nt sequence from Germany (AJ810287), (Ch China, Fr France, Gr Germany, US USA and Ir Iran).

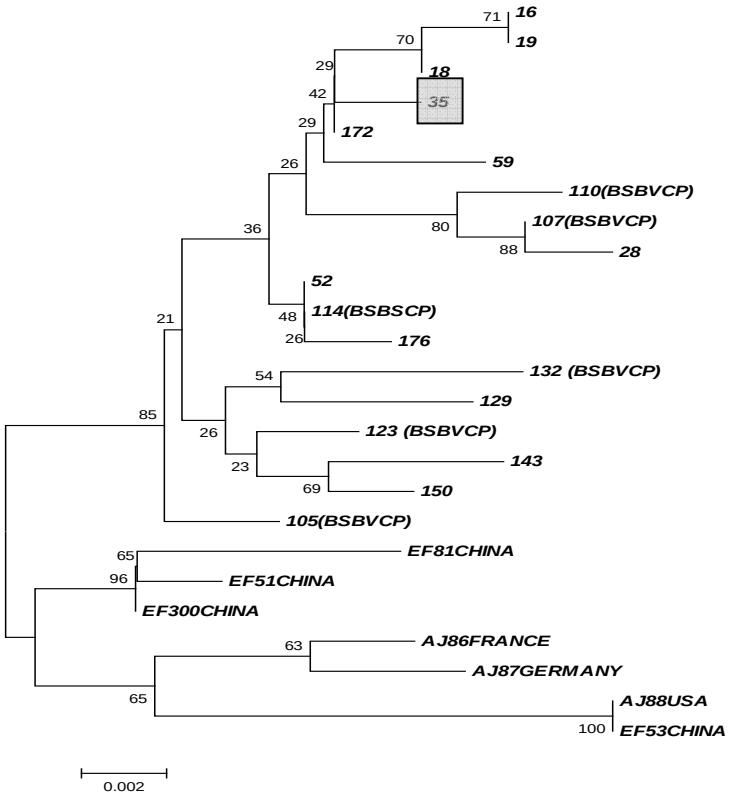


Fig. 2 Phylogenetic tree of the 18 Iranian BSBV coat protein nt sequences plus seven BSBV coat protein nt sequences from Germany, France, USA, and China using MEGA4 program (Neighbor joining method). The gray boxed isolate (Nyshabour, Khorasan Razavi province) fully sequenced.

Making full-length infectious clone of BSBV-Ny isolate from Iranian BSBV sources and its characterization

By making a full-length clone of Iranian BSBV we checked it for the possible infections. This full-length was infectious in *C. quinoa* and showed necrotic local lesions after 5-6 dpi (Fig. 3). So it shows that this virus also has some extend of impact (pathogenicity) and role in rhizomania disease but the exact role and possible interaction of it should be further investigated.

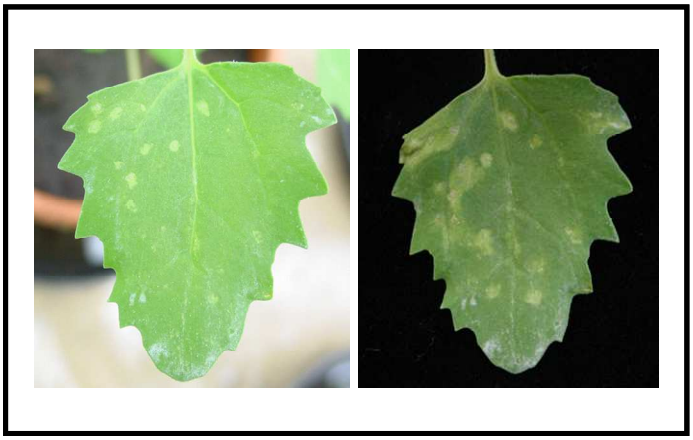


Fig. 3 (**left**) Symptoms of Iranian BSBV-Ny full-length infectious clone on leaves of *C. quinoa* 6 dpi (**right**) and 8 dpi.

Distribution and characterization of Iranian *Beet virus Q*

Beet virus Q was present in 17.2 % (thirty five out of two hundred and three samples) of the Iranian samples. In nine samples (4.1 %) it was accompanied by BNYVV and just in one sample it was accompanied by BBSV (0.46 %). In other cases it was accompanied by two viruses of BNYVV and either BSBV or BBSV. In 3.7 % of the samples BVQ found associated with BNYVV, BSBV and BBSV which raised the question of synergism or recombination amongst these viruses, yet to be investigated. Interestingly also, BVQ was never found altogether with the BBSV satellite, and this fact might be related with the hypothesis of BVQ being possibly a satellite virus, as raised from work of Meunier *et al*. (2005) and Crutzen *et al*. (unpublished data).
In terms of symptoms related to this virus there was not specific symptoms but only root proliferation and general yellowing of the fields. This virus was present in most of the Iranian provinces

cultivated by sugar beet but in a less extend than BNYVV, BSBV and BBSV.

Coat protein sequencing of eleven Iranian BVQ

Eleven isolates of Iranian BVQ out of 34 samples were selected from different parts of Iran and their coat proteins were sequenced. The CP sequences of Iranian BVQ isolates showed not too much variation and most of the genomes of this gene were conserved. These were 14 mutations out of 501 nt. (2.79 % variation in nucleotides level) in positions of 28, 30, 39, 69, 93, 102, 234, 260, 261, 343, 345, 422, 425 and 429 of BVQ CP gene (Fig. 4). But in CP amino acids sequences of 11 BSBV plus two isolates from Germany and France there are just 5 changes (3% variation in amino acid level) (Fig. 4). This showed that most of the mutations like BSBV are silent and the CP sequence is conserved may be due to constraints which imposed by vector or host plants factors.
A phylogenetic tree of 11 nt sequences of Iranian BVQ coat protein, two CP nt sequences from Germany and France and one CP nt sequences of PMTV from Sweden has been shown (Fig. 5).

BVQ Sources	Position number in the coat protein coding region													
	28	30	39	69	93	102	234	260	261	343	345	422	425	429
Ir-141	C	C	U	**C**	G	U	A	**C**	**U**	G	C	A	**G**	A
Ir-145	C	C	U	A	G	U	A	**C**	**U**	G	C	**G**	A	A
Ir-203	C	C	U	A	G	U	A	U	G	G	C	A	A	A
Ir-192	C	C	U	A	G	U	A	U	G	G	C	A	A	A
Ir-189	C	C	U	A	G	U	A	U	G	G	C	A	A	A
Ir-169	C	C	U	A	G	U	A	C	G	G	C	A	A	A
Ir-168	C	C	U	A	G	U	A	U	G	G	C	A	A	A
Ir-159	C	C	U	A	G	U	A	U	G	G	C	A	A	A
Ir-150	C	C	U	A	G	U	A	U	G	G	C	A	A	A
Ir-115	C	C	U	A	G	U	A	U	G	G	C	A	A	A
Ir-136	**G**	C	U	A	G	U	A	U	G	G	**G**	A	A	A
Fr	**A**	C	U	A	G	**C**	A	U	G	G	C	A	A	A
Gr	**A**	**U**	**C**	A	**A**	**C**	**G**	U	G	**A**	C	A	A	**G**

Fig. 4 Comparison of nt sequences of the coat protein of 11 Iranian BVQ isolates by their German (Gr) and French (Fr) counterparts. Numbers are corresponding to coat protein nt sequence of one BVQ isolate from France (AJ810289).

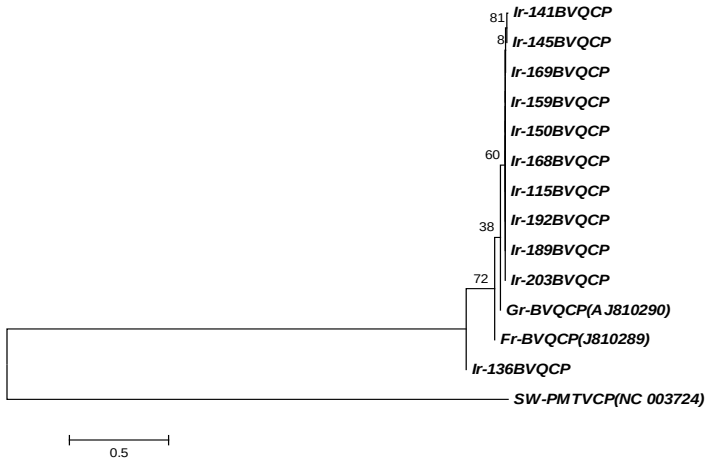


Fig. 5 Phylogenetic tree of 11 Iranian, one German and one French BVQ CP nt sequences plus one nt sequences of PMTV CP from Sweden using MEGA4 program (Neighbor joining method). (Gr Germany, Fr France, SW Sweden)

Chapter V.

Beet black scorch virus satellite is linked with a single Adenine within the virus 3’ untranslated region and contain a partial Argonaute sequence

Journal of general virology (short communication)

Beet black scorch virus satellite is linked with a single Adenine within the virus 3' untranslated region and contain a partial Argonaute sequence

M. Mehrvar & C. Bragard

M. Mehrvar, C. Bragard (author for correspondence)
Université catholique de Louvain, unité de phytopathologie, Louvain-la-Neuve, Belgium
e-mail: claude.bragard@uclouvain.be, 003210/47.37.43. (phone) 003210/47.86.97 (fax)

M. Mehrvar
Ferdowsi University of Mashhad, Faculty of Agriculture, Department of Plant Protection, Mashhad, Iran
e-mail: mehrvar@um.ac.ir

Abstract
Iran was surveyed for *Beet black scorch virus*. The virus was found in 31% of the sugar beet and soil samples and out of 10 provinces when BBSV satellite was found in only 8.8%. BBSV was systematically found in association with the *Beet necrotic yellow vein virus* and/or other sugar beet pomoviruses. The overall organization of the Iranian BBSV genome closely resembled previously described isolates from China and the USA, with identities level from 88% to almost 100 %. Using full-length infectious clones, it was shown that mutations within the 3'UTR, acting as a BYDV cap-independent translation element, did not affect either the RNA structure or the functions of the virus. All the BBSV supporting a satellite did bear an adenine at the position 3398. The Iranian BBSV satellite sequences, despite numerous variations at sequence level, include a sequence highly homologous to the Argonaute 1 gene of sugar beet.

Beet black scorch virus (BBSV) is a *Necrovirus* of the *Tombusviridae* family (Lommel, 2005), described as causing severe, systemic disease symptoms of black scorched leaves and severe stunting in sugar beet (*Beta vulgaris* L.) in China (Yuan *et al.*, 2006; Weiland *et al.*, 2007). The virus is transmitted efficiently through the soil in a non-persistent manner by zoospores of the chytrid *Olpidium brassicae* (Jiang, 1999). It was first identified in Inner Mongolia, China, in the late 1980s (Liu & Xian, 1995; Zhang, 1996; Li *et al.*, 2008a) and was recently reported from the USA (Weiland *et al*., 2007), Iran (Koenig & Valizadeh, 2008) and Europe (Gonzàles-Vàzquez, 2009) where it did not show black scorching on the leaves, but bore exacerbated symptoms of rhizomania, such as rootlet proliferation (Weiland *et al.*, 2007; Gonzàles-Vàzquez, 2009) Symptomless infections have also been observed in sugar beet (Weiland *et al*., 2007). The virus can be detected in the host plants by ELISA, RT-PCR and by retro-inoculation to *Chenopodium quinoa* (Yuan *et al*., 2006).

BBSV shares the highest sequence identity (61%) with *Tobacco necrosis virus* D (TNV-D) (Coutts *et al*., 1991). Complete sequences of one Chinese (BBSV-N) and one US (BBSV-CO) BBSV isolate comprise seven and eight open reading frames (ORFs), respectively (Yuan *et al.*, 2006; Weiland *et al.*, 2007). Normally, only a single RNA was associated with infections, but a small single-stranded RNA (ssRNA) that is similar to the satellite RNAs (sat-RNA) of other necroviruses was found in isolates from the Xinjiang province in China (Guo *et al*., 2005). The sat-RNA of BBSV belongs to the small linear satellite RNA subgroup, which contains several subviral RNAs associated with helper viruses in the *Tombusviridae* family, including *Turnip crinkle carmovirus* (TCV), *Cymbidium ringspot tombusvirus* (CyRSV), *Tomato bushy stunt tombusvirus* (TBSV) and *Cucumber necrosis tombusvirus* (CuNV). The BBSV sat-RNA genome is similar in size to the satellite RNAs of CyRSV (~0.7 kb) and TBSV (~0.7 kb) (Guo *et al*., 2005). This small RNA multiplied in test plants only when associated with BBSV genomic RNA, either from the same viral isolate or from other sources of BBSV (Guo *et al*., 2005). The presence of sat-RNA resulted in more local lesions on the leaves of *C. amaranticolor* plants than did inoculation with BBSV RNA.

Until now, BBSV has been found in distant sugar beet growing areas (Weiland *et al.*, 2007; Koenig & Valizadeh, 2008; Li *et al.*, 2008a; Gonzàles-Vàzquez, 2009). There are no data publicly available yet on the level of distribution of the virus in those countries, or about its economic importance, although by comparison with BNYVV it

might have strategic importance in the future, especially in warmer areas. The recent discovery of BBSV has also raised the question of its origin. In this study, the widespread occurrence of BBSV, alone or with its satellite, in most sugar beet growing areas of Iran was assessed by bioassays and RT-PCR. Research was focused on the 3'UTR, a genome region acting as a *Barley yellow dwarf virus* cap-independent translation element as well as ending by a poly-A tail substitute acting probably as promoter for the initiation of the negative strand synthesis (Shen & Miller, 2007).

A total of 203 soils and sugar beet samples were gathered and tested for the presence of BBSV. The virus was found in 62 (31%) of the tested Iranian sugar beet fields. The virus is present in the 10 provinces where sugar beet is cultivated: it was recorded in 31% of the samples analyzed, but the satellite was detected in only 8.8%, which is nevertheless much higher than reported from China for BBSV. It was distributed in the Iranian provinces of Ardabil, Azarbijaan Sharghi, Azarbijaan Gharbi, Ghazvine, Hamadan, Kerman, Kermanshah, Khorasan Shomali, Khorasan Razavi and Zanjan, representing most of the areas where sugar beet is grown in the country. The satellite of BBSV was also found in 8.8% of the samples and in the same provinces, except for Azarbijaan Sharghi. The symptoms at field level were similar to rhizomania symptoms, mainly root proliferation. Heavily affected sugar beet fields were recorded (Mehrvar *et al*., 2009). Mixed infections of BBSV isolates were also evident in samples from the Kermanshah (Islam Abad Gharb) province.

In this study, the complete nucleotide sequences and genome organization of eight chosen BBSV sources named Ir-AzGh1, Ir-Ha1, Ir-Ha2, Ir-Ksh3, Ir-Ksh6, Ir-Sh1, Ir-Msh1 and Ir-Ksh1 were determined (accession number FN543469, FN543470, FN543466, FN543467, FN543468, FN543471, FN565520, FN543421) and compared with those of US-Co, Ch-N, -X and TNV-D. The linear alignments of the 3,644-nt genomes of Ir-AzGh1, Ir-Ha1, Ir-Ha2, Ir-Ksh3, Ir-Ksh6, Ir-Sh1, Ir-Msh1 and Ir-Ksh1 with US-Co, Ch-N and Ch-X ranged from 88 % to 100 % identity. The major ORFs predicted from the sequences of the US and Chinese BBSV sequences were conserved in Iranian BBSV (Koenig & Valizadeh, 2008). The ORF designation proposed by Yuan *et al*. (2006) will be used hereafter, except when otherwise stated (Yuan *et al*., 2006). The ORF encoding *p5'* and *p4* proteins reported in Chinese (Ch-N) and US (US-Co) isolates, respectively, were not evidenced in Iranian BBSV sources studied (Koenig & Valizadeh, 2008). A putative ORF not present in the Chinese Ch-N sequenced isolates but in the US US-Co isolate, designated as *p10* by Weiland *et al*. (2007), was also found in three

Iranian BBSV. This protein *p10* (designated *p10* in Ir-Ha2, Ir-Ksh3, Ir-Ksh6 and *P9* in Ir-AzGh1) has a start and stop codon ($A_{2693}UG$ to $U_{2977}GA$) in Ir-Ha2, Ir-Ksh3, Ir-Ksh6 and ($A_{2693}UG$ to $U_{2929}GA$) in Ir-AzGh1, which is different from their US counterpart ($A_{2691}UG$ to $U_{2973}GA$). The Iranian Ir-Ha1, Ir-AzGh1 Ir-Sh1, Ir-Msh1 and Ir-Ksh1 isolates contain also a putative gene called *p5b* which was not evidenced in the US and Chinese BBSV isolates. The comparison of the genome sequences of eight BBSV isolates shows a clustering of the strains in two different groups (data not shown), suggesting multiple contamination of sugar beet in Iran, perhaps from another unknown host linked to the soil vector *Olpidium*.

BBSV lacks both a 5' cap and a poly (A) tail, but it is translated efficiently, owing partly to a *Barley yellow dwarf virus* (BYDV)-like cap-independent translation element in its 3' region (UTR) (Shen & Miller, 2007). The analysis of BBSV diversity in Iran reveals a wider diversity than that reported for China or the USA in the portion of the genome. The 3' UTR shows 56 mutations at the nucleotide level, and up to 26 different sequences have been indexed from our isolate collection (Fig 1, accession number for Iranian sequence : FN543402, FN543403, FN543404, FN543405, FN543406, FN543407, FN543408, FN543409, FN543410, FN543411, FN543412, FN543413, FN543414, FN543415, FN543416, FN543417, FN543418, FN543419, FN543420, FN543421, FN543422, FN543423, FN543424, FN543425, FN543426). Mutations did not affect the overall structure of the 3'UTR nor the sites involved in either the canonical BYDV-Cap independent translation element or the poly-A tail substitute potentially involved in the replication initiation (Fig 2). Furthermore, using three different full-length clones of Ir-Ksh1, Ir-Msh1 and Ir-Sh1, it was shown that, despite several mutations, the virus remained infectious and proved functional for both coat protein production as demonstrated by Western blot and replication as verified by real-time RT-PCR (data not shown).

Approximately 25% of the BBSV-containing samples found in Iran are associated with the virus satellite. This satellite has been found to enhance symptom expression, although the symptoms observed were mostly similar to rhizomania symptoms, rather than the beet black scorch symptoms (Weiland *et al.*, 2007; Li *et al.*, 2008a). Weiland *et al.* (2007) reported similar results in the USA, although the sugar beet in their trials was not contaminated by BNYVV.

All the BBSV strains associated with the BBSV satellite clustered together in similar groups when comparing the 3'UTR. This part of the genome is of the uttermost importance for viral replication

and translation, with its *Barley yellow dwarf virus* (BYDV)-like cap-independent translation element, as well as a conserved structure common with other necroviruses (Shen & Miller, 2007). Further analysis is needed to fully understand the reason of this clustering, which might explain the differences observed between satellites from China and Iran. Interestingly, each isolate associated with the satellite has an Adenine instead of a Guanine at the position 3398 (Fig 1).

The four Iranian BBSV satellite sequences, Ir-Ha1sat, Ir-Msh1sat, Ir-Kh1sat and Ir-Bj1sat are constituted of 617 nt, while the previously described Chinese BBSV satellites Ch-Xsat and Ch-2sat isolates have only 615 nt and Ch-3sat has 616 nt with no coding ORF. The comparison between these seven satellite sequences showed up to 116 mutations (Fig. 3a). In a very few cases, the BBSV satellite was found alone without the presence of the helper virus, possibly 'waiting' for the helper virus, as noted by Murant and Mayo (1982). On Iranian BBSV satellite isolates, putative genes possibly coding for small proteins tentatively called *P6* and *P7* have been found, but their real expression or roles have yet to be established. Interestingly, a 47 nt sequence homologous to the *Pisum sativum* Argonaute gene was identified within the P6. It was shown that such a sequence was even more homologous to the AGO sequences from *C. quinoa* (81%) and *Beta vulgaris* (79%) (This study: accession numbers FN556159, FN556160).

The presence of two putative genes within the BBSV satellite raises questions about the role of such peptides as mentioned for TNV (Bringloe *et al.*, 1998). Similarly, the discovery of a piece of sequence homologous to the Argonaute 1 gene of sugar beet raises might indicate that BBSV satellite is targeting the post-transcriptional gene silencing of the host plant for silencing it by its own mechanisms, thus adding another possible suppression route to the already numerous routes listed for plant viruses (Gossele *et al.*, 2002; Wang *et al.*, 2004; Jones *et al.*, 2006; Bortolamiol *et al.*, 2008; Sun *et al.*, 2009).

For the satellite sequence as for the 3'UTR, despite numerous mutations, conserved elements among the viral genome triggers the interest. Yuan *et al*. (2006) hypothesized that stem-loop structures found both at the 5' and 3' end of the satellite might be involved in 5'-3' RNA-RNA interaction, though no protein are encoded by the Chinese BBSV satellite. The potential presence of small ORF within the satellite sequence might explain the presence of such translational enhancers, though structural analysis does not fully support this hypothesis (Guo *et al.*, 2005). Interestingly, the overall structure of the BBSV satellites was found deeply conserved, with a highly ordered

secondary structure made of 32 short inverted repeats of 3 to 9 nucleotides. This structure is probably promoting an efficient replication re-initiation process. It is also interesting to note the conserved 3'end nucleotides, somewhat similar to the 3' end of the BBSV genome, with a GxTxCCC sequence involved in the replication initiation process.

The presence of other soil-borne viruses in co-infection with the BBSV and its satellite was also checked by RT-PCR using the method proposed by Meunier *et al.* (2003). It was found that BBSV was never occurring alone in Iran, but always in co-infections with *Polymyxa*-transmitted soil-borne viruses. It is associated mainly with BNYVV alone or in combination with the sugar beet-infecting pomoviruses, *Beet soil-borne virus* and *Beet virus Q* (Mehrvar *et al.*, 2009). This association might explain the root beardedness observed and also noted by Weiland *et al.* (2007) and Koenig and Valizadeh (2008). The occurrence of up to four different soil-borne viruses also raises questions about the potential synergism or competition between such viruses.

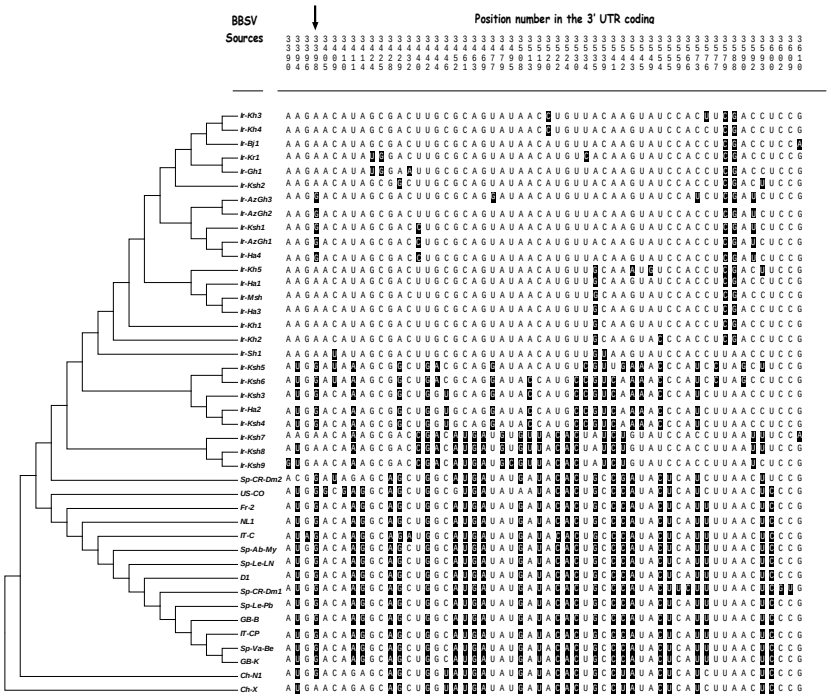


Fig. 1 Evolutionary relationships of the 3' UTR regions of BBSV from various parts of the world, computed by MEGA4 software (Saitou & Nei, 1987; Tamura *et al.*, 2007). The letters preceding the isolates name indicate the country of origin (Ch China, D Denmark, F France, GB Great Britain, Ir Iran, IT Italy, NL Netherlands, Sp Spain, US United States of America.

The GeneBank accession numbers for 3' UTR RNA sequences of BBSV are FN543402, FN543403, FN543404, FN543405, FN543406, FN543407, FN543408, FN543409, FN543410, FN543411, FN543412, FN543413, FN543414, FN543415, FN543416, FN543417, FN543418, FN543419, FN543420, FN543421, FN543422, FN543423, FN543424, FN543425, FN543426, AM941660, AM941659, AM1661, AM941662, AM941663, AM941658, AM941669, AM941670, AM941664, AM941665, AM941668, AM941666 and AM941667 for 3'UTR of isolates Ir-AzGh3, Ir-Ha4, Ir-Ha3, Ir-AzGh2, Ir-Ksh2, Ir-Kr1, Ir-Gh1, Ir-Kh2, Ir-Kh3, Ir-Kh1, Ir-Bj1, Ir-Kh5, Ir-Ksh7, Ir-Kh4, Ir-Ksh8, Ir-Ksh4, Ir-Ksh5, Ir-Ksh9, Ir-Msh, Ir-Ksh1, Sp-Le-Pb, Sp-Le-LN, Sp-Va-Be, Sp-CR-Dm1, CR-Dm2, Sp-Ab-My, D1, NL1, GB-B, GB-K, Fr-2, IT-C, IT-CP, respectively; FN543466, FN543467, FN543468, FN543469, FN543470, FN543471, AF452884, AY626780 and EF153268 for complete RNA sequences of BBSV isolates Ir-Ha2, Ir-Ksh3, Ir-Ksh6, Ir-AzGh1, Ir-Ha1, Ir-sh1, Ch-N1, Ch-X, US-CO respectively.

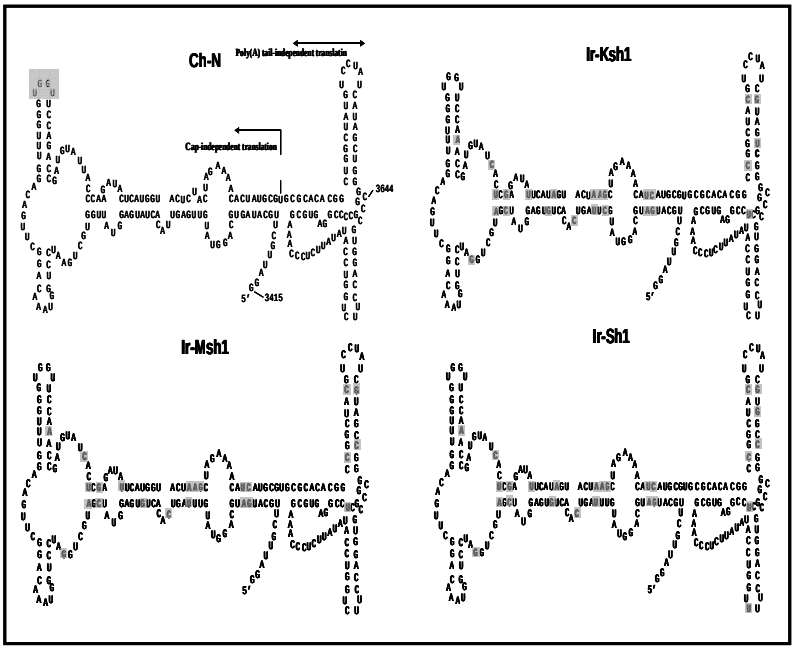


Fig. 2 Comparison of secondary structure and mutations of 26 nt in 3' UTR of the Iranian Ir-Ksh1, Ir-Sh1, and 23 mutations in Ir-Msh1 isolates (associated with BBSV satellite as) compared to the Chinese BBSV-N isolate. Mutations are shown as bold letters. Note the conservation of both the BYDV cap-independent translation element as well as the poly-A tail 3' end substitute involved in replication initiation.

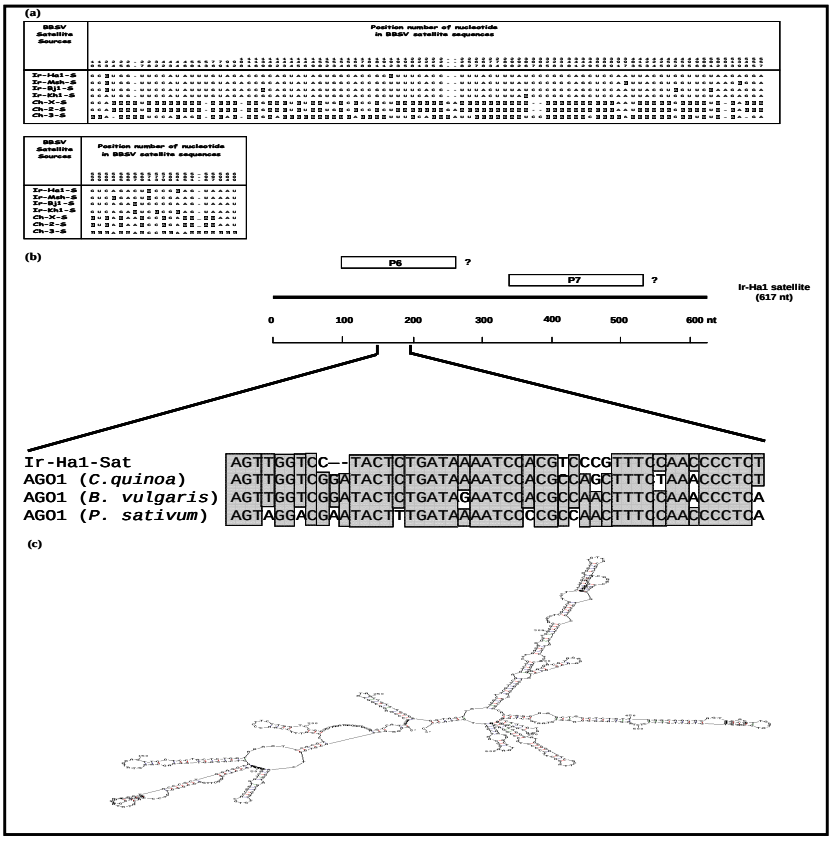


Fig. 3 Comparison of the nucleotide sequences of Iranian BBSV satellite. (a) Comparison of nt sequences of four Iranian BBSV satellites isolates with their Chinese BBSV satellites counterparts. The GeneBank accession numbers for complete RNA sequences of BBSV satellites are FN543472, FN543473, FN543474, FN543475, NC_006460, AY394497 and FJ176575, for isolates Ir-Ha1sat, Ir-Msh1sat, Ir-Kh1sat, Ir-Bj1sat, Ch-Xsat, Ch-2sat, Ch-3sat respectively. (b) Comparison of nt sequences of the Iranian Ir-Ha1 isolate satellite (149-195 nt) with four different Argonaute 1 sources. A 47 nt sequence showed up to 81 % sequence similarity in nt level to *C. quinoa* Argonaute 1 in 49 nt stretch, (Accession no of Argonaute 1 gene for *Chenopodium quinoa*, *Beta vulgaris* and *Pisum sativum* are FN556159, FN556160 and EF108450 respectively). (c) Conserved overall secondary structure of Ir-Ha1sat, obtained by using MFOLD web server interface (version 2.3) (Zuker, 2003) as well as by comparison with the structure proposed by Guo *et al*. (2005).

References

Bortolamiol, D., Pazhouhandeh, M. & Ziegler-Graff, V. (2008). Viral suppression of RNA silencing by destabilisation of ARGONAUTE 1. *Plant Signal Behav* **3**, 657-9.

Bringloe, D. H., Gultyaev, A. P., Pelpel, M., Pleij, C. W. & Coutts, R. H. (1998). The nucleotide sequence of satellite tobacco necrosis virus strain C and helper-assisted replication of wild-type and mutant clones of the virus. *J Gen Virol* **79 (Pt 6)**, 1539-46.

Coutts, R. H., Rigden, J. E., Slabas, A. R., Lomonossoff, G. P. & Wise, P. J. (1991). The complete nucleotide sequence of tobacco necrosis virus strain D. *J Gen Virol* **72 (Pt 7)**, 1521-9.

Gonzàles-Vàzquez, M., Ayala, J., Garcia –Arenal, F. & Fraile, A. (2009). Occurrence of Beet Black scorch virus infecting sugar beet in Europe. *Plant Dis.* **93**, 21-24.

Gossele, V., Fache, I., Meulewaeter, F., Cornelissen, M. & Metzlaff, M. (2002). SVISS - a novel transient gene silencing system for gene function discovery and validation in tobacco plants. *Plant J* **32**, 859-66.

Guo, L. H., Cao, Y. H., Li, D. W., Niu, S. N., Cai, Z. N., Han, C. G., Zhai, Y. F. & Yu, J. L. (2005). Analysis of nucleotide sequences and multimeric forms of a novel satellite RNA associated with beet black scorch virus. *J Virol* **79**, 3664-74.

Jiang, J.-X., Zhang, J.-F., Che, S.-C., Yang, D.-J., Yu, J.-L., Cai, Z.-N. & Liu, Y. (1999). Transmission of beet black scorch virus by Olpidium brassicae. *J Jiangxi Agric Univ* **21**, 525-528.

Jones, L., Keining, T., Eamens, A. & Vaistij, F. E. (2006). Virus-induced gene silencing of argonaute genes in Nicotiana benthamiana demonstrates that extensive systemic silencing requires Argonaute1-like and Argonaute4-like genes. *Plant Physiol* **141**, 598-606.

Koenig, R. & Valizadeh, J. (2008). Molecular and serological characterization of an Iranian isolate of Beet black scorch virus. *Arch Virol* **153**, 1397-400.

Li, J., Li, M., Li, C., Gao, Y., Li, D., Han, C. & Yu, J. (2008). Effects on the local symptoms of subgenomic RNAs expressions and their translational products of Tobacco necrosis virus A Chinese isolate. *Chinese Science Bulletin* **53**, 1682-1690.

Liu, J. & Xian, H. (1995). Preliminary report on Beet black scorch virus. *China Sugar Beet* **3**, 30-31.

Lommel, S. A., Martelli, G. P., Rubino, L. & Russo, M. (2005). Tombusviridae. In *Virus Taxonomy. Eighth Report of the International Committee on Taxonomy of Viruses*, pp. 906–936. Edited by M. A. M. C. M. Fauquet, J. Maniloff, U. Desselberger & L. A. Ball. Lodon: Elsevier/Academic Press.

Mehrvar, M., Valizadeh, J., Koenig, R. & Bragard, C. G. (2009). Iranian beet necrotic yellow vein virus (BNYVV): pronounced diversity of the p25 coding region in A-type BNYVV and identification of P-type BNYVV lacking a fifth RNA species. *Arch Virol* **154**, 501-6.

Meunier, A., Schmit, J. F., Stas, A., Kutluk, N. & Bragard, C. (2003). Multiplex reverse transcription-PCR for simultaneous detection of beet necrotic yellow vein virus, Beet soilborne virus, and Beet virus Q and their vector Polymyxa betae KESKIN on sugar beet. *Appl Environ Microbiol* **69**, 2356-60.

Murant, A. & Mayo, M. (1982). Satellites of plant viruses. *Annu Rev Phytopathol* **20**, 49-70.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406-25.

Shen, R. & Miller, W. A. (2007). Structures required for poly(A) tail-independent translation overlap with, but are distinct from, cap-independent translation and RNA replication signals at the 3' end of Tobacco necrosis virus RNA. *Virology* **358**, 448-58.

Sun, Q., Choi, G. H. & Nuss, D. L. (2009). A single Argonaute gene is required for induction of RNA silencing antiviral defense and promotes viral RNA recombination. *Proc Natl Acad Sci U S A* **106**, 17927-32.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596-9.

Van Regenmortel, M. H. V., Fauquet, C. M., Bishop, D. H. L., Carstens, E., Estes, M. K., Lemon, S., McGeoch, D., Wickner, R. B., Mayo, M. A., Pringle, C. R. & Maniloff, J. (2000). Virus taxonomy. Seventh report of the International Committee on Taxonomy of Viruses, pp. 1025–1032. New York, N.Y.: Academic Press.

Wang, M. B., Bian, X. Y., Wu, L. M., Liu, L. X., Smith, N. A., Isenegger, D., Wu, R. M., Masuta, C., Vance, V. B., Watson, J. M., Rezaian, A., Dennis, E. S. & Waterhouse, P. M. (2004). On the role of RNA silencing in the pathogenicity and evolution of viroids and viral satellites. *Proc Natl Acad Sci U S A* **101**, 3275-80.

Weiland, J. J., Van Winkle, D., Edwards, M. C., Larson, R. L., Shelver, W. L., Freeman, T. P. & Liu, H. Y. (2007). Characterization of a U.S. Isolate of Beet black scorch virus. *Phytopathology* **97**, 1245-54.

Yuan, X., Cao, Y., Xi, D., Guo, L., Han, C., Li, D., Zhai, Y. & Yu, J. (2006). Analysis of the subgenomic RNAs and the small open reading frames of Beet black scorch virus. *J Gen Virol* **87**, 3077-86.

Zhang, K.-W., Wang, S.-L., Cai, Z.-N. & Zhang, H.-S. (1996). Preliminary study on beet black scorch disease in Ningxia Province. *China Sugar Beet* **2**, 3-8.

Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* **31**, 3406-15.

Chapter VI.

A role for BBSV coat protein as symptom and avirulence determinant

A role for BBSV coat protein as symptom and avirulence determinant

M. Mehrvar, & C. Bragard

M. Mehrvar, C. Bragard
Université catholique de Louvain, unité de phytopathologie, Louvain-la-Neuve, Belgium

M. Mehrvar
Ferdowsi University of Mashhad, Faculty of Agriculture, Department of Plant Protection, Mashhad, Iran.

Author for correspondance : claude.bragard@uclouvain.be

Abstract

Beet black scorch virus (BBSV) is a *Necrovirus*, *Tombusviridae*. It was identified at first based on severe black scorching symptoms on sugar beet plant in China but not in Iran, Europe and USA. In order to evaluate the pathogenicity of different isolates of BBSV on sugar beet as well as on other host plants, three different full-length infectious clones were constructed. Two were able to cause black scorching symptoms on the leaves of sugar beet cv. Julietta by mechanical inoculation while the third did not, though they differed only in the coat protein by two different aa. The black scorching ability of sugar beet of the last BBSV source was reestablished by the suppression of the coat protein. The study point out the potential role of aa changes within the coat protein gene in the disease symptom production, as well as the differential reactions observed in different sugar beet cultivars. BBSV CP is therefore proposed as an avirulence determinant based on its role in the differential symptom expression on the different sugar beet cultivars or *Beta macrocarpa* tested. The suppression of the CP gene did also suppressed the systemic mosaic symptom on *Nicotiana benthamiana*, suggesting another role of CP for long distance movement, as previously shown for *Tobacco necrosis virus*.

Introduction

Beet black scorch virus is a soil-borne sugar beet virus, vectored by *Olpidium brassicae* and is a *Necrovirus* of the *Tombusviridae* family (Russo *et al.*, 1981; White & Nagy, 2004). It was first identified in

Inner Mongolia, China, in the 1980s (Liu & Xian, 1995; Zhang, 1996; Li *et al.*, 2008a). Recently it was reported from US (Weiland *et al*., 2007), Iran (Koenig & Valizadeh, 2008) and different European countries (Gonzàles-Vàzquez, 2009). The virus shows proliferation of roots and black scorching of sugar beet leaves in China but the BBSV isolates reported from US, Europe and Iran did not elicit such a black scorching in leaves of sugar beet (*Beta vulgaris* L.). This virus apparently exacerbates symptoms of rhizomania disease of sugar beet like rootlet beardedness (Weiland *et al*., 2007).

The BBSV as a *Necrovirus* of *Tombusviridae* comprises an icosahedral particle of 28 nm that encapsidates a positive-sense, single-stranded (ss) genomic (g) RNA of 3644 nucleotides. BBSV shares the highest sequence identity (61%) with *Tobacco necrosis virus* D (TNV-D) (Coutts *et al*., 1991). Complete sequences of two Chinese (BBSV-N, -X) and one US (BBSV-Co) BBSV isolates showed they have seven and eight open reading frames (ORFs) respectively.

The *P23* and *P82* ORFs are thought to encode the viral RNA polymerase, as the P82 protein contains a GDD motif that is conserved in RNA-dependent RNA polymerases (Argos, 1988). The P82 ORF, residing between nucleotides 36 and 2210, is probably expressed directly from the gRNA via translational readthrough of an amber stop codon. p23 may have a role in targeting replication complex to the cellular membrane in cytoplasm (Weiland *et al*., 2007).

The small ORF of *P5'*, *P7a* and *P7b* ORFs, located in the central region of the BBSV RNA genome, are involved in cell-to-cell movement of the virus in host plants. Each of these genes is required for localized movement, accumulation of viral RNAs and formation of local lesions on the leaves of *Chenopodium amaranticolor*, as it was evidenced for similar proteins encoded by subgenomic (sg) RNA in TNV-D (Coutts *et al*., 1991) and TNV-DH required for cell-to-cell movement (Drouzas, 1996; Molnar *et al.*, 1997; Yuan *et al.*, 2006).

The 39-proximal ORF (nt 2647–3345) in BBSV is predicted to encode the coat protein (*p24 - CP*). The coat proteins of TNV-DH (Molnar *et al*., 1997) and *Turnip crinkle virus* have been demonstrated to be involved in long-distance movement during systemic infection of *Nicotiana benthamiana*, but in BBSV no information is yet reported. Cao *et al.* (2006) showed that leaves of *C. amaranticolor* inoculated with *in vitro* transcripts of the mutant in which the entire CP gene was deleted had lower local lesions than wild type virus transcripts which indicate that coat protein does not necessarily have any effect on virus replication in *C. amaranticolor* (Cao *et al.*, 2006).

It is reported that two sgRNAs along with the viral gRNA are present in plant tissues infected with BBSV, and these sgRNAs are not encapsidated in viral particles (Guo *et al*., 2005).
This study was initiated with the view of understanding the molecular determinants of the black scorch symptom, by using selected BBSV sources (chapter 5), combined with a strategy of reverse genetic applied to the virus coat protein gene.

Materials and methods

Bait plant technique

After all stones and visible roots were removed from the soil samples, each sample was ground. Four days germinated sugar beet seeds of the rhizomania susceptible cultivar Cadyx were planted into 300 ml plastic pots containing each of the soil samples mixed with sterile sand (1: 4 V/V). Then, 200 ml of Hoagland solution was added to each pot. Two replicate pots of each soil were placed in a controlled environment room at 19 ºC (night) and 22 ºC (day) with a 16 h photoperiod. The bait plants were removed from the soil after eight weeks and their rootlets were carefully washed in running tap water, cut and stored at minus 20 ºC until used. Uninfected seedlings were grown under the same conditions in sand-sterile soil mixture as a control (Meunier *et al*., 2003).

Mechanical inoculation

Plants rootlets were washed and ground in 0.01 M sodium phosphate buffer, pH 7.0. The extract inoculated in upper side of *Chenopodium quinoa* leaves mechanically. Test of host range performed by transfer of virus from infected *C. quinoa* to the leaves of *Beta macrocarpa*, *Nicotiana benthamiana*, *Spinacia oleracea* and sugar beet (*Beta vulgaris*) cv. Cadyx, Sofia and Julietta, using mechanical inoculation as aforementioned.

Molecular characterization

Full length genome of Iranian BBSV

Total RNA was extracted from healthy and infected *C. quinoa* leaves using a SV Total RNA Extraction Kit (Promega). The Expand Reverse Transcriptase kit (Roche), which includes a high-fidelity, thermo-stable polymerase, was used to generate a complete BBSV genome-length amplicon using Primer designated BBT75fwd and BBSV3rev (Weiland *et al*., 2007). The amplicons were cloned into

pDrive vector (Qiagene, Germany). EcoR1 digestion of clones liberated the clone inserted from the plasmid vector, providing a template from which transcription was initiated with one G nucleotide before the viral sequence.

Transcription and inoculation of construct on *C. quinoa*, sugar beet, spinach and *N. benthamiana*

Transcription reaction with T7 RNA polymerase for generating uncapped RNA was used to produce genome-length RNA for inoculation to plant leaves. Released construct was transcripted by T7 RiboMAX™ Express Large Scale RNA Production System (promega). *In vitro* transcripts was mixed in the inoculation buffer - 2 to 5 µg of RNA in GKP buffer (50 Mm glycine, 30 mM K2HPO4, 1% bentonite, and celite, pH 9.2) and mechanically inoculated on leaves of *C. quinoa*, *N. benthamiana*, *B. macrocarpa*, sugar beet and *S. oleracea*) (Weiland & Edwards, 1994).

Making BBSV-Ksh1 mutant lacking coat protein

The coat protein of BBSV-Ksh1 construct was suppressed by overlap PCR. The primers which used for suppression of BBSV-Ksh1 construct CP were BBT75fwd and BBSV3rev which was reported by Weiland *et al*. (2007) plus BBmut14F AAgGGCACCTAAACGCAACA (nt positions 2646-2665) and BBmut14R TGTTGCGTTTAGGTGCCcTT (nt positions 2665-2646) primers were used for construction of the BBSV-Ksh1ΔCP mutant in 3 round of overlapping PCR. In BBSV-Ksh1 construct the nt U in position 2648 was substituted by nt G in order to switched off the CP of BBSV-Ksh1ΔCP mutant (Fig. 6a).

Western blot

The CP of BBSV-Ksh1, BBSV-Msh1, BBSV-Sh1 constructs but not BBSV-Ksh1ΔCP mutant were detected by western blotting after SDS-PAGE separation of total protein extracts from symptomatic leaves of *C. quinoa*. Structural proteins were revealed using a polyclonal antibody directed against viral particles (kindly provided by Prof. Winter, DSMZ). The primary antibody was diluted 1:2000 in TBS buffer containing 0.1 % Tween 20, 1 % powdered skimmed milk, and supplemented with total protein extracts from healthy *C. quinoa*. Anti rabbit alkaline phosphatase-conjugated secondary IgG (Sigma-Aldrich) was diluted 1:5000 in TBS-Tween buffer and the blots were revealed with premixed BCIP-NBT solution (Sigma-Aldrich) (Fig. 4).

RT-qPCR

A set of primer plus its TaqMan probe was designed using different sources of BBSV as a reference. This could successfully amplify a fragment of 80 bp in p7a gene of BBSV which is conserved in all of BBSV isolates. The forward and reverse primers which started and stopped in nt positions 2201 and 2281 respectively were TGGGGCATGAAAACCTAACC and GTCCTCCGATCTGCTTCTCA and TaqMan probe was TCACGACGCTGTTCACTACGCTGT at nt position 2259 of BBSV nt sequence which is within p7a gene. According to our previous results (chapter 5) in most of to date known sequences of BBSV, the p7a gene is conserved.

Results

The recent discovery of BBSV in numerous and different sugar beet growing areas worldwide like China, Europe, Iran and U.S.A. (Liu & Xian, 1995; Zhang, 1996; Koenig & Valizadeh, 2008; Li *et al.*, 2008a) (Gonzàles-Vàzquez, 2009), sometimes associated with strong symptoms and yield impact raises concern about such virus.
Three BBSV isolates have been selected based on their differences (chapter 5): a source from Kermanshah (West of Iran), named Ir-Ksh1, and not associated with the BBSV satellite, another source from Mashhad, called Ir-Msh1 and a third one from Shirvan named Ir-Sh1, both being associated with a BBSV satellite and located North-East of the country. The three different virus sources did not show any black scorching symptoms on the leaves of sugar beet in the fields, but were all associated with BNYVV the causal agent of rhizomania disease (Mehrvar *et al.*, 2009) as well as with symptoms of root proliferation. Each of the three virus sources produced necrotic local lesions on C. quinoa 3 dpi. The RNA was extracted from these necrotic local lesions of symptomatic leaves of *C. quinoa* in all three samples and the presence of BBSV was confirmed by sequencing of RT-PCR product. Full-length infectious clone for each of the three selected isolates produced similar necrotic local lesion as wild type isolates on *C. quinoa* 3 dpi.
The two Iranian isolates of BBSV-Msh1 and BBSV-Sh1 caused black scorching on leaves of sugar beet (cv. Julietta) and the symptoms were more severe in BBSV-Sh1 isolate (Fig. 1). These symptoms appeared 5 dpi like black colored necrotic lesions in leaves of inoculated sugar beet and extended for 3-4 weeks to total collapse of the inoculated leaves. These isolates were not systemic in sugar beet. Interestingly the black scorching symptoms were shown to be host-dependant. In sugar beet cv. Cadyx and Sofia, only pinpoint necrotic local lesions evolving into larger yellow coloured chlorotic local lesions were

observed, and the black scorching symptoms did not appeared. The BBSV CP is also needed for infection of spinach (*S. oleracea*) as the BBSV-Ksh1ΔCP mutant, unable to produce the BBSV coat protein, did not cause any symptoms on spinach.
Interestingly BBSV-Ksh1ΔCP mutant showed black scorching symptoms in sugar beet (Julietta) (Fig. 5) while the BBSV-Ksh1 did not (Fig. 2C). This indicates that the coat protein of BBSV is able to trigger the resistance response of the host plants for this isolate. Nevertheless, other isolates like BBSV Ir-Sh-1 or Ir-Msh-1 were still able to trigger the black scorching reaction without suppression of the CP, indicating that minor changes at the amino acid level might trigger the occurrence of the severe black scorching symptoms on sugar beet (Fig 2).
In fig. 6b, the comparison of aa sequences of three different Iranian BBSV coat protein gene with other sources of BBSV from China and USA has been shown. The differences between pathogenic (BBSV-Msh1, BBSV-Sh1) and non pathogenic (BBSV-Ksh1) isolates of BBSV on sugar beet is possibly due to the minor changes at the amino acid positions 12, 145 and 158 of the BBSV coat protein gene. Of particular interest is the aa at the position 12 where a nonpolar aa is modified to a polar amino acid (I12T) for a change to a virulent isolate, but with the question still remaining of a different aa lying within the Chinese isolate able to cause black scorching, therefore explained by the context. Further investigations considering the BBSV folding, based on the TNV CP model (Oda *et al.*, 2000), might deliver an answer to this question.

Discussion

Coat proteins of many different plant viruses play multifunctional roles. They function in every aspect of viral multiplication and dissemination, with roles for assistance in replication of the viral nucleic acid, movement between cells and organs, and travel from infected to uninfected plants via mobile biological vectors. In the constant dynamic struggle between host and parasite, plants have often evolved to recognize the viral CPs as elicitor molecules of host defense machinery, presumably providing the host with an early warning system against an impending viral invasion (Callaway *et al.*, 2001).
An important aspect of disease induction is the ability of a plant virus to overcome the defensive strategies employed by the host. One mechanism of disease resistance is the hypersensitive response (HR), a cascade of signal transduction events that eventually lead to necrosis and cell death at sites of infection and thus prevent spread of the pathogen (Callaway *et al.*, 2001). In *Turnip crinkle virus* (TCV) of the

family *Tombusviridae* and TNV, the coat protein is responsible for viral recognition and induction of resistance responses as for the symptom modulation of virus infection (Wang & Simon, 2000; Zhao *et al.*, 2000). TCV CP was also showed to function as an elicitor for the hypersensitive response (HR) to virus infection in *Arabidopsis* in which alteration of the amino acids at N-terminal position of 4 and 85 led to breaking of virus resistance in *Arabidopsis* ecotype Di-17 (Zhao *et al.*, 2000). Coat proteins of TNV-DH *(*Molnar *et al*., 1997) and TCV have been demonstrated to be involved in long-distance movement during systemic infection of *N. benthamiana*. TCV coat protein function also as a RNA silencing suppressor (Qu *et al.*, 2003; Thomas *et al.*, 2003). The coat protein of *Cymbidium ringspot tombusvirus* (CymRSV) was recognized as an *avirulence* factor as it induces a very rapid HR-like response of *Datura stramonium* after virus infection (Szittya & Burgyan, 2001). Li *et al.* (2008) by suppression of coat protein of TNV-A showed that the intact TNV-AC coat protein was dispensable for establishment of TNV-AC infection in *C. amaranticolor*, with numbers of local lesions and the viral RNA accumulation level reduced, or the time to symptom appearance significantly delayed in case of coat protein suppression (Li *et al.*, 2008a). In BBSV it was showed that inoculated leaves of *C. amaranticolor* with *in vitro* transcripts of the mutant in which the entire CP gene was deleted had lower local lesions than wild type virus transcripts which indicate that coat protein does not necessarily have any effect on virus replication in *C. amaranticolor* (Cao *et al.*, 2006).

Suppression of Iranian BBSV-Ksh1 coat protein led in a similar manner to a reduced replication of RNA genome and larger but much less necrotic local lesions on *C. quinoa* leaves, as well as also no symptoms on spinach inoculated leaves (Fig 3C). Like for TNV-D (Molnar *et al.*, 1997), BBSV without CP was unable to cause systemic infections on *N. benthamiana.*

CP as the elicitor interacts directly with either the *R* gene product or another factor in the signal transduction pathway. Consequently, the interaction occurs at one or a few amino acids, often on the surface of the CP. Alteration of the key amino acid(s) either alters its specificity as an elicitor or abolishes the elicitation altogether. In addition, a single CP can act as elicitor for different *R* genes in different hosts (Callaway *et al.*, 2001).

This finding could use for the search for future resistance against BBSV in breeding program.

Fig. 1 Induction of black scorching symptoms in leaves of *Beta vulgaris* (Julietta) by Iranian BBSV-Sh1 construct 5, 6, 9, 10 and 14 dpi respectively (left to the right).

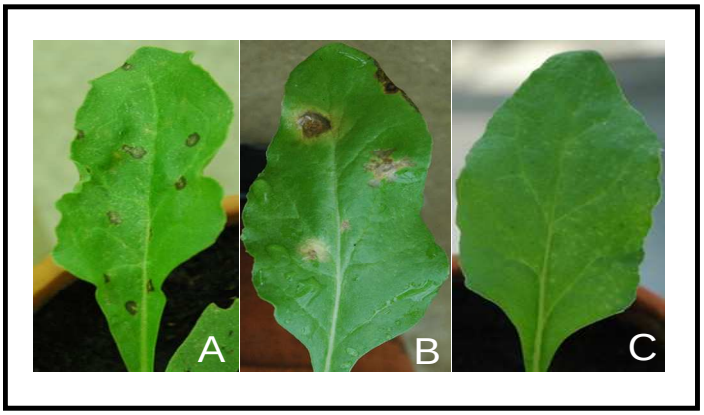


Fig. 2 Induction of black scorching symptoms by Iranian BBSV-Sh1 construct (5 dpi) (**A**), BBSV-Msh1 construct (20 dpi) (**B**) and necrotic local lesions by BBSV-Ksh1 construct (20 dpi) (**C**) on *Beta vulgaris* (Julietta).

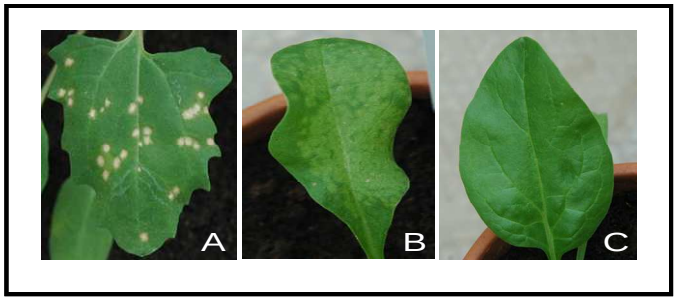


Fig. 3 Induction of necrotic local lesions in *Spinacia oleracea* (**B**) and *Chenopodium quinoa* (**A**) inoculated leaves 5 dpi by BBSV-Ksh1 constructs but not *Spinacia oleracea* (**C**) by BBSV-Ksh1ΔCP mutant (5dpi).

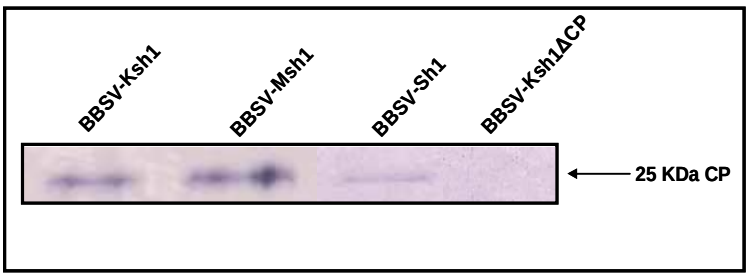


Fig. 4 Western blot analysis for the detection of structural proteins of Iranian BBSV-Ksh1, BBSV-Msh1, BBSV-Sh1 constructs and BBSV-Ksh1ΔCP mutant in *C. quinoa* leaves 5dpi. The 25 KDa CP of BBSV-Ksh1, BBSV-Msh1and BBSV-Sh1 constructs but not BBSV-Ksh1ΔCP mutant were immuno-detected by a polyclonal antisera raised against viral particle

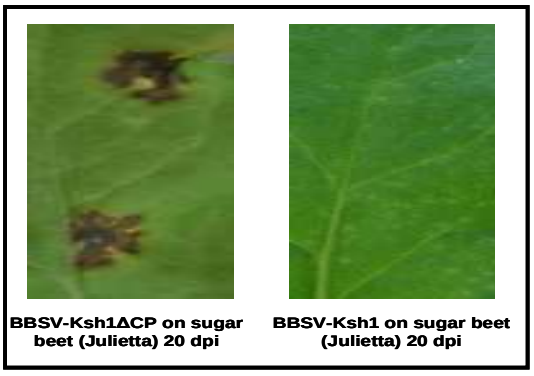


Fig. 5 Induction of black scorching symptoms by BBSV-Ksh1ΔCP mutant (**left**) and necrotic local lesions by BBSV-Ksh1constructs (**right**) on *Beta vulgaris* (Julietta) leaves 20 dpi.

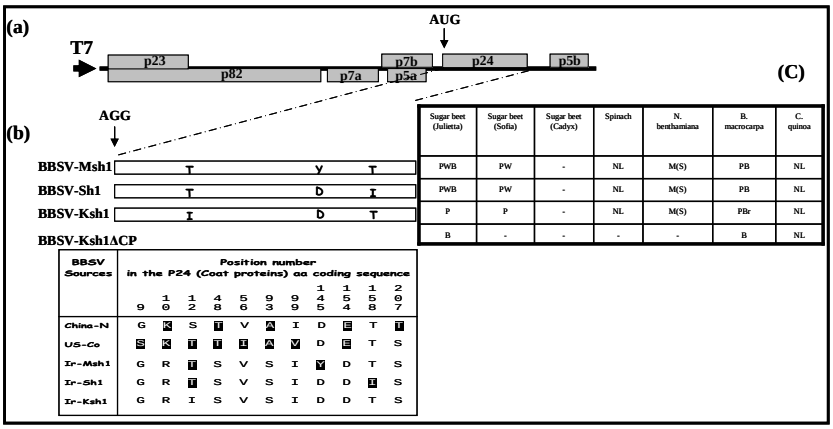


Fig. 6 (**a**) Organization of BBSV-Ksh1 genome and its mutant for coat protein gene suppression (created by a single change in CP start codon in position of 2648 of BBSV-Ksh1construct nt sequence), (**b**) Comparison of aa sequences of the coat protein of three Iranian isolates of BBSV-Msh1, BBSV-Ksh1 and BBSV-Sh1by their US (BBSV-Co) and Chinese (BBSV-N) counterparts. Numbers are corresponding to aa in coat protein of BBSV from China, US and Iran. (Accession numbers for BBSV-Co (US isolate) and BBSV-N (Chinese isolate) are EF153268 and AF452884 respectively), (**C**) Manifestation of different symptoms on different host plants by BBSV-Msh1, BBSV-Sh1 and BBSV-Ksh1 constructs plus BBSV-Ksh1ΔCP mutant inoculated mechanically on leaves of these plants (P (pinpoint local lesions), W (White color local lesion), B (Black scorching), NL (Necrotic local lesions), M (Mosaic, S (Systemic) and Br (Brown local lesions).

References

Argos, P. (1988). A sequence motif in many polymerases. *Nucleic Acids Res* **16**, 9909-16.

Callaway, A., Giesman-Cookmeyer, D., Gillock, E. T., Sit, T. L. & Lommel, S. A. (2001). The multifunctional capsid proteins of plant RNA viruses. *Annu Rev Phytopathol* **39**, 419-60.

Cao, Y., Yuan, X., Wang, X., Guo, L., Cai, Z., Han, C., Li, D. & Yu, J. (2006). Effect of *beet black scorch virus* coat protein on viral pathogenicity. *Progress in Biochemistry and Biophysics* **33**, 127-134.

Coutts, R. H., Rigden, J. E., Slabas, A. R., Lomonossoff, G. P. & Wise, P. J. (1991). The complete nucleotide sequence of tobacco necrosis virus strain D. *J Gen Virol* **72 (Pt 7)**, 1521-9.

Drouzas, A. D., Offei, S. K. & Coutts, R. H. A. (1996). Subcellular location and expression of tobacco necrosis necrovirus p7a protein. *J Phytopathol* **144**, 297-299.

Gonzàles-Vàzquez, M., Ayala, J., Garcia –Arenal, F. & Fraile, A. (2009). Occurrence of Beet Black scorch virus infecting sugar beet in Europe. *Plant Dis.* **93**, 21-24.

Guo, L. H., Cao, Y. H., Li, D. W., Niu, S. N., Cai, Z. N., Han, C. G., Zhai, Y. F. & Yu, J. L. (2005). Analysis of nucleotide sequences and multimeric forms of a novel satellite RNA associated with beet black scorch virus. *J Virol* **79**, 3664-74.

Koenig, R. & Valizadeh, J. (2008). Molecular and serological characterization of an Iranian isolate of Beet black scorch virus. *Arch Virol* **153**, 1397-400.

Li, J., Li, M., Li, C., Gao, Y., Li, D., Han, C. & Yu, J. (2008). Effects on the local symptoms of subgenomic RNAs expressions and their translational products of Tobacco necrosis virus A Chinese isolate. *Chinese Science Bulletin* **53**, 1682-1690.

Liu, J. & Xian, H. (1995). Preliminary report on Beet black scorch virus. *China Sugar Beet* **3**, 30-31.

Mehrvar, M., Valizadeh, J., Koenig, R. & Bragard, C. G. (2009). Iranian beet necrotic yellow vein virus (BNYVV): pronounced diversity of the p25 coding region in A-type BNYVV and identification of P-type BNYVV lacking a fifth RNA species. *Arch Virol* **154**, 501-6.

Meunier, A., Schmit, J. F., Stas, A., Kutluk, N. & Bragard, C. (2003). Multiplex reverse transcription-PCR for simultaneous detection of beet necrotic yellow vein virus, Beet soilborne

virus, and Beet virus Q and their vector Polymyxa betae KESKIN on sugar beet. *Appl Environ Microbiol* **69**, 2356-60.

Molnar, A., Havelda, Z., Dalmay, T., Szutorisz, H. & Burgyan, J. (1997). Complete nucleotide sequence of tobacco necrosis virus strain DH and genes required for RNA replication and virus movement. *J Gen Virol* **78 (Pt 6)**, 1235-9.

Oda, Y., Saeki, K., Takahashi, Y., Maeda, T., Naitow, H., Tsukihara, T. & Fukuyama, K. (2000). Crystal structure of tobacco necrosis virus at 2.25 A resolution. *J Mol Biol* **300**, 153-69.

Qu, F., Ren, T. & Morris, T. J. (2003). The coat protein of turnip crinkle virus suppresses posttranscriptional gene silencing at an early initiation step. *J Virol* **77**, 511-22.

Russo, M., Martelli, G. P. & Di Franco, A. (1981). he fine structure of local lesions of beet necrotic yellow vein virus in Chenopodium amaranticolor. *Physiological Plant Pathology* **19**, 237-242.

Szittya, G. & Burgyan, J. (2001). Cymbidium ringspot tombusvirus coat protein coding sequence acts as an avirulent RNA. *J Virol* **75**, 2411-20.

Thomas, C. L., Leh, V., Lederer, C. & Maule, A. J. (2003). Turnip crinkle virus coat protein mediates suppression of RNA silencing in Nicotiana benthamiana. *Virology* **306**, 33-41.

Wang, J. & Simon, A. E. (2000). 3'-End stem-loops of the subviral RNAs associated with turnip crinkle virus are involved in symptom modulation and coat protein binding. *J Virol* **74**, 6528-37.

Weiland, J. J. & Edwards, M. C. (1994). Evidence that the alpha a gene of barley stripe mosaic virus encodes determinants of pathogenicity to oat (Avena sativa). *Virology* **201**, 116-26.

Weiland, J. J., Van Winkle, D., Edwards, M. C., Larson, R. L., Shelver, W. L., Freeman, T. P. & Liu, H. Y. (2007). Characterization of a U.S. Isolate of Beet black scorch virus. *Phytopathology* **97**, 1245-54.

White, K. A. & Nagy, P. D. (2004). Advances in the molecular biology of tombusviruses: gene expression, genome replication, and recombination. *Prog Nucleic Acid Res Mol Biol* **78**, 187-226.

Yuan, X., Cao, Y., Xi, D., Guo, L., Han, C., Li, D., Zhai, Y. & Yu, J. (2006). Analysis of the subgenomic RNAs and the small open reading frames of Beet black scorch virus. *J Gen Virol* **87**, 3077-86.

Zhang, K.-W., Wang, S.-L., Cai, Z.-N. & Zhang, H.-S. (1996). Preliminary study on beet black scorch disease in Ningxia Province. *China Sugar Beet* **2**, 3-8.

Zhao, Y., DelGrosso, L., Yigit, E., Dempsey, D. A., Klessig, D. F. & Wobbe, K. K. (2000). The amino terminus of the coat protein of Turnip crinkle virus is the AVR factor recognized by resistant arabidopsis. *Mol Plant Microbe Interact* **13**, 1015-8.

Chapter VII. Discussion

At the beginning of this research, the knowledge on soil-borne sugar beet in Iran was only limited: the presence of the BNYVV had been mentioned, but its distribution was unknown as well as its diversity. A major contribution of this work is to acknowledge the large occurrence of four different telluric sugar beet viruses: the BNYVV, the *Pomovirus* BSBV and BVQ and the *Necrovirus* BBSV and their distribution (Fig 6).

The table 1 shows the percentage of occurrence of each virus combination found. It emphasize that such distribution is certainly not random, but probably responding to rules linked to the need for other viruses acting as « helper » or « assistant », either for the soil-borne transmission by the vector *Polymyxa betae* or for the establishment of the virus in the plant. Except BNYVV able to stand alone in approximately 30 % of the collected samples, most of the studied viruses were found in association with at least another virus. For example, if BNYVV was found in combination with either pomoviruses and/or BBSV, the combination of BBSV (*Necrovirus*) with either BSBV or BVQ was never found, raising questions about the reasons for such an absence while their relative frequencies is probably not sufficient for explaining the phenomenon. Interestingly also, BVQ was never found altogether with the BBSV satellite, and this fact might be related with the hypothesis of BVQ being possibly a satellite virus, as raised from work of Meunier *et al.* (2005) and Crutzen *et al.* (unpublished).

There is a real need for studying the different interactions between each of the virus species described here. But before starting such a work, a more in-depth knowledge is a pre-requisite. It’s the reason why each of the viruses was studied one by one. It’s also the reason why we decided to contribute to the construction of full length clones which will be useful in reverse genetic approaches for studying the virus-virus or virus-vector interactions.

Therefore, we will discuss each of the results virus by virus, for ending by a general view on how to link the present knowledge on soil-borne viruses in Iran with the strategies that could be used to limit their spread and effect on sugar beet yield.

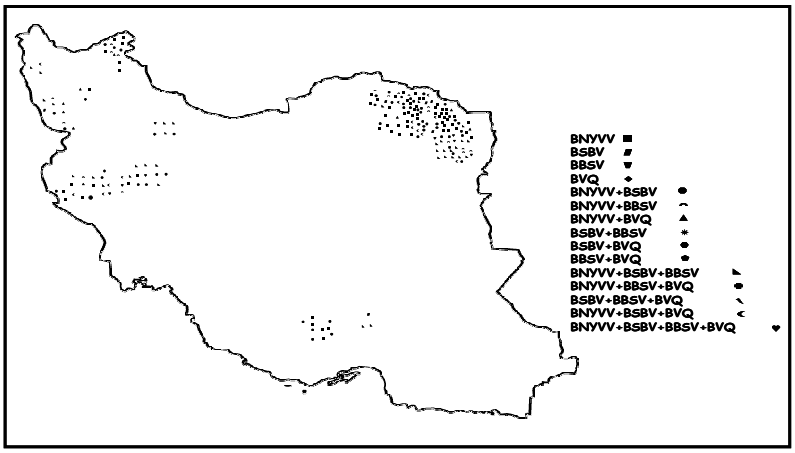


Fig. 6 The map of distribution of BNYVV, BSBV, BVQ and BBSV or a mixture of them in different parts of Iran.

No	Virus	Percentage %
1	BNYVV	65 (30 %)
2	BSBV	6 (2.7 %)
3	BNYVV+BSBV	50 (23.1 %)
4	BNYVV+BBSV	16 (7.4 %)
5	BNYVV+BVQ	9 (4.1 %)
6	BBSV+BVQ	1(0.46 %)
7	BNYVV+BSBV+BBSV	31 (14.35 %)
8	BNYVV+BBSV+BVQ	5 (2.31 %)
9	BNYVV+BSBV+BVQ	12 (5.5 %)
10	BNYVV+BSBV+BBSV+BVQ	8 (3.7 %)

Table 1 The table of percentage of single or mixed infection of four soil-borne sugar beet viruses in Iran.

VII.1. Iranian *Beet necrotic yellow vein virus* (BNYVV)

Up to now, sequence information is mainly available for European, American and East Asian BNYVV isolates. The present study provides a comprehensive overview of the distribution of various BNYVV types in Iran.

Molecular analysis of the four RNA species always present in natural BNYVV infections have revealed the existence of four major strain groups of the virus named A, B, J and P types (Kruse *et al.*, 1994; Koenig *et al.*, 1995; Koenig & Lennefors, 2000; Schirmer *et al.*, 2005; Ratti *et al.*, 2006; Ward *et al.*, 2007). The three types A, B and P were found to be highly conserved in Europe and recently determined sequences are practically identical to those determined more than two decades ago by Bouzoubaa *et al.* (Bouzoubaa *et al.*, 1985; Bouzoubaa *et al.*, 1986; Bouzoubaa *et al.*, 1987). BNYVV sources from East Asian countries (Japan, China) are similar to the European A and B types, but not completely identical to them (Miyanishi *et al.*, 1999; Koenig & Lennefors, 2000; Meunier *et al.*, 2005; Ward *et al.*, 2007).

In Iran mostly the BNYVV A-type was found. The amino acid tetrad in position 67 – 70 of the RNA 3-encoded p25 in Iranian A type BNYVV showed considerable variation. Altogether nine different variants were identified. The diversity observed within the p25 tetrad is the highest observed in the world for such an area. By comparison, a similar study on BNYVV diversity gathering samples from six Chinese sugar beet-growing regions evidenced seven different tetrad motifs (Li *et al.*, 2008b) also a study in Turkey showed the dominant presence of some of these tetrads (Kutluk Yilmaz *et al.*, 2007). Interestingly also, the proportions of different p25 tetrads observed is totally different from other areas where such data have already been gathered, as in Western Europe or USA (Schirmer *et al.*, 2005; Liu & Lewellen, 2007). Possibly their occurrence was due to spontaneous mutations as suggested by Acosta-Leal and Rush (2007) (Acosta-Leal & Rush, 2007). An explanation for this might be the wide use of non-resistant polygerm sugar beet cultivars in conjunction, in some areas, with the intense use of monogerm tolerant ones, with the possibility of raising soil infectious potential to high levels and then confronting such soils with virus resistance genes.

p25 encoded on RNA-3 enables the systemic movement of the virus in sugar beet and is mainly responsible for symptom expression (Tamada *et al.*, 1989; Koenig *et al.*, 1991; Jupin *et al.*, 1992) and root proliferation in sugar beet (Tamada *et al.*, 1999). Its ability to shuttle between the nucleus and the cytoplasm is necessary for symptom expression on leaves of *Chenopodium quinoa* (Vetter *et al.*, 2004). The amino acid tetrad in positions 68 to 70 of P25 has been found to be highly variable in BNYVV sources collected at many different

places in the world (Tamada *et al.*, 2002; Koenig *et al.*, 2005; Meunier, 2005; Schirmer *et al.*, 2005; Ratti *et al.*, 2006; Ward *et al.*, 2007; Li *et al.*, 2008b). Chiba *et al.* (2002; 2008) found that the resistance response of selected lines of *Beta maritima* and the sugar beet variety Rizor depended on the composition of this tetrad. p25 was thus identified as an avirulence factor in these resistances (Chiba *et al.*, 2002; Chiba *et al.*, 2008). Klein *et al.* (2007) found that sequence variations of p25 influenced its oligomerization and pathogenicity in *Tetragonia expansa* (Klein *et al.*, 2007). Acosta-Leal and Rush (2007) identified $V_{67}E_{135}$ in p25 as the signature for resistance breaking variants of BNYVV from the Imperial Valley in the US as opposed to the WT motif $A_{67}D_{135}$ (Acosta-Leal & Rush, 2007). Other variants of the tetrad have also been associated with resistance breaking (Rush *et al.*, 2006; Liu & Lewellen, 2007).

In Iran BNYVV P-type was found too in a distantly areas. The SYHG-containing p25 of the Iranian P type had an identical amino acid composition in all 23 samples investigated. They originated from different areas in the North East, the North West and West of Iran. This P type was found preferentially in fields heavily cropped with sugar beet resistant varieties. The amino acid composition of this Iranian P type p25 was very similar to that of P type isolates from other countries (France, Kazakhstan, and UK) and to that of the SYHG-containing p25 from two isolates in the USA.

In none of the investigated fields was BNYVV RNA-5 was found. RNA-5 is normally associated with P type BNYVV in France (Koenig *et al.*, 1997b; Schirmer *et al.*, 2005), Kazakhstan (Koenig & Lennefors, 2000) and the UK (Harju *et al.*, 2002; Ward *et al.*, 2007). RNA 5-free P type has, however, previously been reported from a location in France (Lemaire *et al.*, 2003).

The presence of a fifth RNA species in the BNYVV genome in some areas has also been associated with an increased BNYVV pathogenicity of the virus (Tamada *et al.*, 1996a). In East Asia BNYVV RNA-5 has been found to be frequently associated with A or B type-like BNYVV (Li *et al.*, 1999; Miyanishi *et al.*, 1999). A distinct type of RNA5 has been found to be associated with P type BNYVV, a virus type which so far has been detected only in France, Kazakhstan and more recently in the UK (Koenig *et al.*, 1997b; Koenig & Lennefors, 2000; Harju *et al.*, 2002; Ward *et al.*, 2007).

This study demonstrates the occurrence of wide variations within the RNA-3-located p25 coding region in a vast country like Iran. Looking at studies on the impact of such variations on resistance response (Chiba *et al.*, 2002; Chiba *et al.*, 2008) and pathogenicity (Acosta-Leal & Rush, 2007; Klein *et al.*, 2007; Liu & Lewellen, 2007), it stresses the need for developing efficient strategies for controlling a wide variety of different types and subtypes of BNYVV.

The presence of different subtypes of BNYVV A in Iran possibly due to extensive use of susceptible local sugar beet varieties, sometimes in alternance with tolerant sugar beet varieties like (Laetitia, Universal, Paulina, Dorotea, Pekora and …).
Also, favorable conditions for the rhizomania disease to expand are all present in Iran: short term rotations with cereals, use of improper methods for irrigation, susceptible varieties and simply conducive temperature and humidity conditions.
The presence of BNYVV P-type in fields of sugar beet in Iran and especially in fields grown with tolerant varieties reinforces the hypothesis that the virus might evolve under the varieties selection pressure in order to survive. Nevertheless, up to now, there is no clear evidence to support the idea that the virus is evolving by loosing its RNA-5 to become a P-type without its fifth RNA, or if the virus is mutating within the RNA-3 sequence to resemble to other reported P-types. If the occurrence of P-types is deriving from a multipartite virus with 5 RNA segments, its presence in different areas in Iran is suggesting multiple contaminations from different sources.

VII.2. Iranian sugar beet pomoviruses

As Iranian sugar beet fields are heavily contaminated by BSBV (47%) and in a lesser extend by BVQ (16.7%), the question of the impact of such viruses on sugar beet crop alone or together with BNYVV is of major importance. Heavily infected fields by all sugar beet soil-borne viruses BNYVV, BSBV and BVQ have been spotted in Iran.
Using an Iranian BSBV source, a full-length infectious clone was built (Crutzen *et al.*, 2009b) for starting a reverse genetic approach. By using such strategy it was possible to demonstrate the ability of the virus to cause symptom on *Chenopodium* as well as the dispensability of coat protein in the pathogenicity.
There is a genuine need to investigate the exact impact of these viruses on their hosts. In terms of BVQ, it was accompanied by BBSV just in one occasion. In most of the cases it was found together with BNYVV. The relative low rate of BVQ incidence in Iran (16%) support the hypothesis that it is less competent than BSBV and BBSV, may due to its dependence to BNYVV or its transmission to host plant by *P. betae*.
Interestingly the level of sequence homology between Iranian *Pomovirus* and sequences from other geographical origin was high. This emphasizes the fact that compared to BNYVV which shows a lot of variation, these viruses appears as more stable. Taking into account the fact that both studied pomoviruses are equally transmitted by *P. betae*, this observation reinforce the idea that BNYVV is evolving

under the pression of the sugar beet genotypes resistant to the benyviruses, on the contrary of the pomoviruses.
The exact impact of pomoviruses on sugar beet has not been yet known but they are mostly accompanied by BNYVV and sometimes by BBSV and in all of cases the infected samples show the proliferation of tap roots which is not clear yet pomoviruses take part in inducing such a symptoms or not but with use of infectious clone of BSBV from Iran. Following this thesis, it is possible now to investigate the impact of BSBV alone, or in conjunction with BNYVV or BBSV on sugar beet.
The co-occurrence of so many soil-borne viruses, sharing a similar vector and host plant, raises also the question of potential synergism or recombination speeding up their evolution and adaptation to sugar beet genotypes. If such similar viruses do occur simultaneously within a single cell, they might, at least theoretically, recombine or exchange genome RNA segments. If there is no evidence for this up to date, the hypothesis might not be excluded either. But the absence of such events might also be explained by a possible compartmentalization of the different viruses within the plant root.

VII.3. Iranian *Beet black scorch virus* (BBSV)

For the first time, a new emerging soil-borne virus was found on sugar beet in Iran. Such a discovery raises the question once again of a synergistic effect of a complex of all of these soil-borne viruses or not? In this study, more than 60 BBSV sources and 18 BBSV satellite sources have been identified and partially characterized, allowing us to select up to seven different and well-differentiated sources from different geographical localization for further and more in-depth studies.
BBSV was reported previously from four different distant parts of the world China, US and most recently from Europe (Fig. 9) (Weiland *et al.*, 2007; Li *et al.*, 2008a; Gonzàles-Vàzquez, 2009).
The Iranian BBSV is widespread in most of sugar beet fields. For the first time in Iran it was found in Khorasan Razavi province in North-East, a place near to Xinjiang province in China where for the first time BBSV was found in the world in 1980s. Interestingly in both places the BBSV hold a satellite RNA. Also the Iranian BBSV is more diverse than other sources of BBSV from China, USA and Europe may be due to presence of other host plant such as *Brassicaceae* which can easily infected by the vector and could serve as a source for variation as well as reservoirs for this virus in rotation period.
Also the recombination between different isolates of BBSV or between BBSV and other necroviruses like TNV should be further investigated to know that they play a role in so many variations in

Iranian BBSV or not as the mixed infection of different isolates of BBSV was evidenced in some parts of Iran.
In terms of BBSV symptoms it reported that it can cause black scorching in sugar beet leaves and proliferation in sugar beet tap roots in China but the BBSV reported from Iran, Europe and USA did not show such black scorching of leaves and just proliferation of tap roots mostly accompanied by BNYVV the causal agent of rhizomania disease.
The presence of satellite can exacerbate the symptoms of disease in indicator plants *Chenopodium quinoa* which may be due to presence of a fragment of 47 nt which is more than 80 % similar to a fragment of 49 nt of Argonaute 1 gene of this plant and may be involve in the suppression of gene silencing.
The exact impact of this virus on sugar beet has not yet been elucidated but the pathogenicity of this virus when it was accompanied by BNYVV was high and in some cases the fields was abandoned by farmers due to unprofitable crops.
Absence of this virus in south of the country in Fars province may be due a preference of *Olpidium brassicae* for colder climate.
Seven different full-length sequences of BBSV and their satellite from Iran were constructed on this basis. Iranian BBSV sources BBSV-Ha1, BBSV-Msh1 and BBSV-Sh1 were found associated with a satellite comprising 617 nucleotides instead of 615 nt in the previously described Chinese BBSV-X. By using these infectious clones it is possible to evaluate the synergism or antagonist impact of BBSV on BNYVV, BSBV and BVQ in host plants and propose measures to contain all of these viruses at the same time and further investigate the impact of BBSV on host plants alone or in conjunction by other soil-borne sugar beet viruses in the future.
By comparing more than 20 BBSV isolates for their 3' UTR containing a BYDV-cap independent translation element (BYDV-CITE) the major importance of such sequence demonstrated by showing the secondary structure conservation despite numerous nucleotide changes in both parts of 3' UTR cap independent translation element part (CITE) and in poly (A) tail independent translation and replication part (Shen & Miller, 2004, , 2007).
Coat proteins have been reported as multifunctional proteins in many plant viruses. In the constant dynamic struggle between host and parasite, plants have often evolved to recognize viral CPs as elicitor molecules of host defense machinery, presumably providing the host with an early warning system against an impending viral invasion (Callaway *et al.*, 2001). In *Turnip crinkle virus* (TCV) of the family *Tombusviridae* and TNV, the coat protein was responsible for viral recognition and induction of resistance responses and also symptom modulation of virus infection (Wang & Simon, 2000; Zhao *et al.*,

2000), also it showed function as an elicitor for the hypersensitive response (HR) to virus infection in *Arabidopsis* in which alteration of the amino acids at N-terminal position of 4 and 85 led to breaking of virus resistance in *Arabidopsis* ecotype Di-17 (Zhao *et al.*, 2000). Also the coat proteins of TNV-DH (Molnar *et al*., 1997) and *Turnip crinkle virus* have been demonstrated to be involved in long-distance movement during systemic infection of *Nicotiana benthamiana*, also, TCV coat protein function as a RNA silencing suppressor (Qu *et al.*, 2003; Thomas *et al.*, 2003). The coat protein of *Cymbidium ringspot tombusvirus* (CymRSV) was recognized as an *avirulence* factor in very rapid HR-like response of *Datura stramonium* to virus infection (Szittya & Burgyan, 2001). Li *et al*. (2008) by suppression of coat protein of TNV-A showed that the intact TNV-AC coat protein was dispensable for establishment of TNV-AC infection in *C. amaranticolor*, otherwise the numbers of local lesions and the viral RNA accumulation level were reduced, or the time to symptom appearance significantly delayed in case of coat protein suppression (Li *et al*., 2008a). In BBSV it was showed that inoculated leaves of *C. amaranticolor* with *in vitro* transcripts of the mutant in which the entire CP gene was deleted had lower local lesions than wild type virus transcripts which indicate that coat protein does not necessarily have any effect on virus replication in *C. amaranticolor* (Cao *et al.*, 2006), and may be BBSV CP has one of these roles in host plant as it is evidenced its has a role in pathogenicity of virus in host plants.

The coat protein gene of BBSV was suppressed to assess its role in formation of black scorching symptoms. Using such a strategy, it was possible to produce black scorching symptoms with the BBSV-Ksh1 isolate bearing CP suppression. There is a single amino acid changes between BBSV-Ksh1 and other BBSV isolates causing black scorching symptoms. Up to now, we do not know what the exact role of CP in inducing symptoms is, except that CP plays a role also in the long distance movement of the virus and that the symptoms produce by a single infectious clone are different according to the host plant tested. Also the presence of BBSV coat protein is need for systemic infection of *N. benthamiana* as TNV-D (Molnar *et al*., 1997) and TMV (Ding *et al*., 1996) and may a mechanism like TMV is responsible for this phenomenon which showed that this mutant is accumulate in phloem parenchyma cells but is not detected in companion cells and further research revealed that the coat protein in TMV is needed to protect the viral RNA in phloem tissue as well as interacting with host components of the vascular plasmodesmata (Astier *et al.*, 2007).

The BBSV CP is also needed for infection of spinach (*Spinacia oleracea*) and causing symptoms may be virion but not RNA alone is needed for the infection of this host plant possibly due to cell to cell

movement of the virus from plasmodesmata to reach the cell distantly far from initially infected cells because in *C. quinoa* this mutants replicate and causing necrotic local lesion in a greatly reduced manner.

VII.4. Control measures

From the knowledge provided by this work on BNYVV in Iran, the following recommendations could be made for containing the rhizomania disease in the country:

- Use of tolerant varieties in infected fields, following an evaluation of their resistance against the different BNYVV types present in Iran as well as to BBSV.
- Proper sanitary measures like limitation of soil movement between farms
- Use of proper irrigation methods to avoid excessive soil moisture favorable to the vector, *Polymyxa betae*.
- Use of longer rotation periods with plant non-host for BNYVV and *Polymyxa betae*, like cereals
- Interdiction of sowing sugar beet in heavily infected fields, to avoid the build up of aggressive, resistance breaking isolates.
- Holding educational workshops for inspectors and farmers, who can assist in the disease detection and management. Such inspectors should be provided with clear information on the different viruses possibly involved in the disease as well as to the different symptoms that might be expected, for example black

For containing pomoviruses in the first step we should know them and their impact on sugar beet well and then take action. The general control measures of pomoviruses are like containing rhizomania disease because the vector is the same but about using tolerant varieties there is not reported varieties against these viruses up to now.
The widespread presence of BBSV in Iran is due to poor sanitary measures, extensive use of susceptible varieties (Although up to now there is not resistant varieties of sugar beet against this virus), bad irrigation methods like floating fields or furrows with water which can favor the spread of virus by its vector *Olpidium brassicae* and short rotation.
The elimination of weeds of sugar beet fields which possibly may be a reservoir of the virus also can contain the disease.

Chapter VIII. Conclusion

Conclusion

Using a smart sampling strategy for targeting sugar beet fields presenting soil-borne viral diseases, four different telluric viruses BNYVV, BSBV, BBSV & BVQ and in different mixture have been mapped in Iran.

This study allowed verifying the widespread presence of rhizomania in the country like BNYVV type A which is widely spread in Iran with nine different variants in BNYVV RNA3 p25 tetrad position commonly associated with significant damages to the crop.

Furthermore, the presence of the pathotype P (lacking RNA5) in numerous soils is indicative of the presence of aggressive BNYVV isolates.

BNYVV was found to be associated with three other viruses. First of all, and even if their contribution to the etiology remains a matter of debate, pomoviruses like BSBV and in a lesser extend, BVQ, usually associated with rhizomania, have been found quite frequently for a country with warmer areas. Their contribution to the disease remains to be clarified.

This work contributed to the elaboration of BSBV full length clones which shall be used in a reverse genetic strategy to elucidate their role in the pathogenic process alone or in combination with BNYVV or BBSV.

Finally, BBSV was discovered for the first time in Iran. Published in the meanwhile by Koenig and Valizadeh (2008), this work is describing the genetic diversity of this *Necrovirus*, and its satellite. Iranian BBSV has a considerable variation in nt. sequence level in comparison to other sources of BBSV which is a matter of interest for screening of diversity of this virus and sequence divergence with the already known BBSV isolates.

The detection of a sequence homologous to the Argonaute gene 1 and its relative similarity with similar gene lying within the sugar beet genome stress the specific interactions between the virus and its plant host. It is tempting to speculate about a role of such a sequence for suppressing the gene silencing by targeting the core of the system itself, even if such a hypothesis still needs to be verified. Furthermore, it was shown that a single Adenine within the 3'UTR is probably linked to the ability to act concurrently with the satellite.

A major question regarding BBSV is its ability to cause symptoms like black scorching on sugar beet. Here it was shown that the virus is able to cause strong symptoms on several host plants and that specific isolates induced black scorching on common sugar beet cultivars, even those bearing resistance genes to BNYVV. By reverse genetic the role of coat protein and p5a in the formation of symptom of black

scorching was demonstrated. The BBSV coat protein can be targeted for producing BBSV-resistance transgenic sugar beet.

Started from the field's problems and finished at the genome level, this work is the demonstration that numerous major information can be collected and patiently gathered to build a view as faithful as possible of the virus, its satellite or vectors. This type of approach allows to better understand the dynamic of the virus epidemics, in a complementary manner to molecular reverse

This work was contributed to a better knowledge of four different soil-borne viruses of sugar beet in Iran and allows offering new strategies for the control of these viruses in a major crop in the country. It's also a necessary first step before starting on the interaction between these viruses as well as with their vectors.

Chapter IX. References

Abe H & Tamada T (1986). Association of Beet necrotic yellow vein virus with isolates of Polymyxa betae KESKIN. *Ann. Phytopathol. Soc. Japan* **52,** 235-245.

Abe H & Ui T (1986). Host range of Polymyxa betae Keskin strains in rhizomania-infested soils of sugar beet in Japan. *Annals of the Phytopathological Society of Japan* **52,** 394-403.

Acosta-Leal R, Fawley MW & Rush CM (2008). Changes in the intraisolate genetic structure of Beet necrotic yellow vein virus populations associated with plant resistance breakdown. *Virology* **376,** 60-68.

Acosta-Leal R & Rush CM (2007). Mutations Associated with Resistance-Breaking Isolates of Beet necrotic yellow vein virus and Their Allelic Discrimination Using TaqMan Technology. *Phytopathology* **97,** 325-330.

Adams JM, Antoniw JF & Mullins JGL (2001). Plant virus transmission by plasmodiophorid fungi is associated with distinctive transmembrane regions of virus-encoded proteins. *Arch. Virol.* **146,** 1139–1153.

Adams M (1990). Epidemiology of fungally-transmitted viruses. *Soil Use Manag* **6,** 184–189.

Adams MJ (1991). Transmission of plant viruses by fungi. *Annals of Applied Biology* **118,** 479-492.

Andika IB, Kondo H, Rahim MD & Tamada T (2006). Lower levels of transgene silencing in roots is associated with reduced DNA methylation levels at non-symmetrical sites but not at symmetrical sites. *Plant Mol Biol* **60,** 423-435.

Argos P (1988). A sequence motif in many polymerases. *Nucleic Acids Res* **16,** 9909-9916.

Asher M (1993). Rhizomania. In *The Sugar Beet Crop, Science into Practice*. ed. Cooke D & Scott R, pp. 311-346. Chapman & Hall, London.

Asher M (1999). Sugar-beet rhizomania: the spread of a soilborne disease. *Microbiol. Today* **26,** 120-122.

Asher MJC, Chwarszczynskam DM & Leaman M (2002). The evaluation of Rhizomania resistant sugar beet for the UK. *Annals of Applied Biology* **141,** 101-109.

Astier S, Albouy J, Maury Y & Lecoq H (2007). *Principles of Plant Virology (Genomes, Pathogenicity, Virus Ecology).* Science publisher.

Balmori E, Gilmer D, Richards K, Guilley H & Jonard G (1993). Mapping the promoter for subgenomic RNA synthesis on beet necrotic yellow vein virus RNA 3. *Biochimie* **75,** 517-521.

Barr D (1992). Evolution and kingdoms of organisms from the perspective of a mycologist. *Mycologia* **84,** 1-11.

Barr K & Asher MJC (1992). The host range of polymyxa betae in Britain. *plant pathology* **41,** 64-68.

Berg JM (1990). Zinc finger domains: hypotheses and current knowledge. *Annu Rev Biophys Biophys Chem* **19,** 405-421.

Bleykasten-Grosshans C, Guilley H, Bouzoubaa S, Richards KE & Jonard G (1997). Independent expression of the first two triple gene block proteins of beet necrotic yellow vein virus complements virus defective in the corresponding gene but expression of the third protein inhibits viral cell-to-cell movement. *Mol Plant Microbe Interact* **10,** 240–246.

Bo Y-X, Cai, Z.-N., Ding, Q., Yu, J.-L. & Liu, Y. (1996). Characterization of beet black scorch virus RNA, partial cDNA cloning and sequencing of the small component. *J Agric Biotechnol* **4,** 269-276.

Bortolamiol D, Pazhouhandeh M & Ziegler-Graff V (2008). Viral suppression of RNA silencing by destabilisation of ARGONAUTE 1. *Plant Signal Behav* **3,** 657-659.

Bos L (1999). *Plant viruses, unique and intriguing pathogens a text book of plant virology.* Backhuys, Leiden.

Boughey CL (1981). Evolution and taxonomic studies in wild and cultivated beets, vol. phD. University of Birmingham, Birmingham, UK.

Bouzoubaa S, Guilley H, Jonard G, Richards K & Putz C (1985). Nucleotide sequence analysis of RNA-3 and RNA-4 of Beet necrotic yellow vein virus, isolates f2 and g1. *J. Gen. Virol.* **66,** 1553-1564.

Bouzoubaa S, Niesbach-Klosgen U, Jupin I, Guilley H, Richards K & Jonard G (1991). Shortened forms of beet necrotic yellow vein virus RNA-3

and -4: internal deletions and a subgenomic RNA. *J Gen Virol* **72 (Pt 2),** 259-266.

Bouzoubaa S, Quillet L, Guilley H, Jonard G & Richards K (1987). Nucleotide sequence of beet necrotic yellow vein virus RNA-1. *Journal of General Virology* **68,** 615-626.

Bouzoubaa S, Ziegler V, Beck D, Guilley H, Richards K & Jonard G (1986). Nucleotide sequence of Beet necrotic yellow vein virus RNA-2. *J. Gen. Virol.* **67,** 1689-1700.

Braselton JP (1995). Current status of the plasmodiophorids. *Crit Rev Microbiol* **21,** 263-275.

Bringloe DH, Gultyaev AP, Pelpel M, Pleij CW & Coutts RH (1998). The nucleotide sequence of satellite tobacco necrosis virus strain C and helper-assisted replication of wild-type and mutant clones of the virus. *J Gen Virol* **79 (Pt 6),** 1539-1546.

Cai Z-N, Chen, D.-H., Wu, M.-S., Cui, X.-M., Yu, J.-L. & Liu, Y. (1993). Identification of pathogenic virus of beet black scorch disease and detection by synthesized cDNA probes. *J Beijing Agric Univ* **19,** 112.

Callaway A, Giesman-Cookmeyer D, Gillock ET, Sit TL & Lommel SA (2001). The multifunctional capsid proteins of plant RNA viruses. *Annu Rev Phytopathol* **39,** 419-460.

Campbell RN (1996). Fungal transmission of plant viruses. *Annu Rev Phytopathol* **34,** 87-108.

Canova A (1959). On the pathology of sugar beet. *Informatore Fitopatologico* **9,** 390-396.

Cao Y, Yuan X, Wang X, Guo L, Cai Z, Han C, Li D & Yu J (2006). Effect of *beet black scorch virus* coat protein on viral pathogenicity. *Progress in Biochemistry and Biophysics* **33,** 127-134.

Carpenter CD, Cascone PJ & Simon AE (1991). Formation of multimers of linear satellite RNAs. *Virology* **183,** 586–594.

Chiba S, Miyanishi M, Andika IB, Kondo H & Tamada T (2008). Identification of amino acids of the beet necrotic yellow vein virus p25 protein required for induction of the resistance response in leaves of Beta vulgaris plants. *J Gen Virol* **89,** 1314-1323.

Chiba S, Miyanishi M & Tamada T (2002). Single amino acid changes in the P25 protein of Beet necrotic yellow vein virus determine resistance responses of Beta vulgaris spp. maritima. In *Proceedings of the 5th symposium of the international working group on plant viruses with fungal vectors*. ed. Rush Cm & Merz U, pp. 5-8, Zurich.

Ciafardini G (1991). Evaluation of Polymyxa betae Keskin Contaminated by Beet Necrotic Yellow Vein Virus in Soil. *Appl Environ Microbiol* **57,** 1817-1821.

Cohen Y, Gisel A & Zambryski PC (2000). Cell-to-cell and systemic movement of recombinant green fluorescent protein-tagged turnip crinkle viruses. *Virology* **273,** 258-266.

Commandeur U, Koenig R, Lesemann DE, Torrance L, Burgermeister W, Liu Y, Schots A, Alric M & Grassi G (1992). Epitope mapping on fragments of beet necrotic yellow vein virus coat protein. *J Gen Virol* **73 (Pt 3),** 695-700.

Commandeur U, Koenig R, Manteuffel R, Torrance L, Luddecke P & Frank R (1994). Location, size, and complexity of epitopes on the coat protein of beet necrotic yellow vein virus studied by means of synthetic overlapping peptides. *Virology* **198,** 282-287.

Cooper JI & Asher MJC (1988). Viruses with fungal vectors. In *Association of Applied Biologists*, Wellesbourne. England.

Coutts RH, Rigden JE, Slabas AR, Lomonossoff GP & Wise PJ (1991). The complete nucleotide sequence of tobacco necrosis virus strain D. *J Gen Virol* **72 (Pt 7),** 1521-1529.

Crutzen F, Kreit M & Bragard C (2009a). The beet virus Q coat protein readthrough domain is longer than previously reported, with two transmembrane domains. *J Gen Virol* **90,** 754-758.

Crutzen F, Mehrvar M, Gilmer D & Bragard C (2009b). Full-length infectious clone of Beet soil-borne virus indicates the dispensability of the RNA-2 for virus survival in planta and symptom expression on Chenopodium quinoa leaves. *J Gen Virol*.

de Biaggi M (1987). Methodes de selection – un cas concret. In *IIRB 50th Winter Cong*, vol. 2, pp. 157-163, Brussels, Belgium.

Dessens JT & Meyer M (1996). Identification of structural similarities between putative transmission proteins of Polymyxa and Spongospora transmitted bymoviruses and furoviruses. *Virus Genes* **12,** 95-99.

Diao A, Chen J, Gitton F, Antoniw JF, Mullins J, Hall AM & Adams MJ (1999). Sequences of European wheat mosaic virus and oat golden stripe virus and genome analysis of the genus furovirus. *Virology* **261,** 331-339.

Ding X, Shintaku MH, Carter SA & Nelson RS (1996). Invasion of minor veins of tobacco leaves inoculated with tobacco mosaic virus mutants defective in phloem-dependent movement. *Proc Natl Acad Sci U S A* **93,** 11155-11160.

Drouzas AD, Offei, S. K. & Coutts, R. H. A. (1996). Subcellular location and expression of tobacco necrosis necrovirus p7a protein. *J Phytopathol* **144,** 297-299.

Dunoyer P, Pfeffer S, Fritsch C, Hemmer O, Voinnet O & Richards KE (2002). Identification, subcellular localization and some properties of a cysteine-rich suppressor of gene silencing encoded by peanut clump virus. *Plant J* **29,** 555-567.

Erhardt M, Dunoyer P, Guilley H, Richards K, Jonard G & Bouzoubaa S (2001). Beet necrotic yellow vein virus particles localize to mitochondria during infection. *Virology* **286,** 256-262.

Erhardt M, Vetter G, Gilmer D, Bouzoubaa S, Richards K, Jonard G & Guilley H (2005). Subcellular localization of the Triple Gene Block movement proteins of Beet necrotic yellow vein virus by electron microscopy. *Virology* **340,** 155-166.

Farzadfar S, Pourrahim R, Golnaraghi AR & Ahoonmanesh A (2007). Surveys of Beet necrotic yellow vein virus, Beet soil-borne virus, Beet virus Q and Polymyxa betae in sugar beet fields in Iran. *J. Plant Pathol.* **89,** 277-281.

Fauquet CM, Mayo MA, Maniloff.J, Desselberger.U & Ball.L.A (2005). *Virus Taxonomy, VIIIth Report of the ICTV*. Elsevier/Academic Press, London.

Frese L, Desprez B & Ziegler D (2001). Potential of genetic resources and breeding strategies for base-broadening in Beta In *Broadening the*

genetic base of crop production. ed. Cooper Hd, Spillane C & Hodgkin.T, pp. 295-309. IPGRI/FAO, Rom.

Gerik JS & Duffus JE (1988). Differences in vectoring ability and aggressiveness of isolates of Polymyxa betae. *Phytopathology* **78,** 1340-1343.

Gilmer D, Allmang C, Ehresmann C, Guilley H, Richards K, Jonard G & Ehresmann B (1993). The secondary structure of the 5'-noncoding region of beet necrotic yellow vein virus RNA 3: evidence for a role in viral RNA replication. *Nucleic Acids Res* **21,** 1389-1395.

Gilmer D, Bouzoubaa S, Hehn A, Guilley H, Richards K & Jonard G (1992a). Efficient cell-to-cell movement of beet necrotic yellow vein virus requires 3' proximal genes located on RNA 2. *Virology* **189,** 40-47.

Gilmer D, Richards K, Jonard G & Guilley H (1992b). cis-active sequences near the 5'-termini of beet necrotic yellow vein virus RNAs 3 and 4. *Virology* **190,** 55-67.

Giltrap N, Baker R & Henry C (2001). Sugar Beet Rhizomania-a technical overview. Techical briefing, Central Science Laboratory (CSL), York. http://www.defra.gov.uk/planth/Rhizback.pdf.

Goffart JP & Maraite H (1992). Influence of temperature on Polymyxa betae and beet necrotic yellow vein virus (BNYVV). *Mededelingen van de Faculteit Landbouwwetenschapen, Rijksuniversiteit Gent* **57**.

Gonzàles-Vàzquez M, Ayala, J., Garcia –Arenal, F. & Fraile, A. (2009). Occurrence of Beet Black scorch virus infecting sugar beet in Europe. *Plant Dis.* **93,** 21-24.

Gossele V, Fache I, Meulewaeter F, Cornelissen M & Metzlaff M (2002). SVISS - a novel transient gene silencing system for gene function discovery and validation in tobacco plants. *Plant J* **32,** 859-866.

Guo LH, Cao YH, Li DW, Niu SN, Cai ZN, Han CG, Zhai YF & Yu JL (2005). Analysis of nucleotide sequences and multimeric forms of a novel satellite RNA associated with beet black scorch virus. *J Virol* **79,** 3664-3674.

Haeberle AM & Stussi-Garaud C (1995). In situ localization of the non-structural protein P25 encoded by beet necrotic yellow vein virus RNA 3. *J Gen Virol* **76 (Pt 3),** 643-650.

Haeberle AM, Stussi-Garaud C, Schmitt C, Garaud JC, Richards KE, Guilley H & Jonard G (1994). Detection by immunogold labelling of P75 readthrough protein near an extremity of beet necrotic yellow vein virus particles. *Arch Virol* **134,** 195-203.

Harju VA, Mumford RA, Blockley A, Boonham N, Clover GRG, Weekes R & Henry CM (2002). Occurrence in the United Kingdom of Beet necrotic yellow vein virus isolates which contain RNA 5. *plant pathology* **51,** 811 - 811.

Harju VA, Skelton A, Clover GR, Ratti C, Boonham N, Henry CM & Mumford RA (2005). The use of real-time RT-PCR (TaqMan) and post-ELISA virus release for the detection of Beet necrotic yellow vein virus types containing RNA 5 and its comparison with conventional RT-PCR. *J Virol Methods* **123,** 73-80.

Hartwright LM, Hunter PJ & Walsh JA (2009). A comparison of Olpidium isolates from a range of host plants using internal transcribed spacer sequence analysis and host range studies. *Mycol Res.*

Harveson R, Rush C & Wheeler T (1996). The spread of *Beet necrotic yellow vein virus from point source inoculation as influenced by irrigation and tillage*. *Phytopathology* **86,** 1242-1247.

Hehn A, Bouzoubaa S, Bate N, Twell D, Marbach J, Richards K, Guilley H & Jonard G (1995). The small cysteine-rich protein P14 of beet necrotic yellow vein virus regulates accumulation of RNA 2 in cis and coat protein in trans. *Virology* **210,** 73-81.

Hehn A, Bouzoubaa S, Jonard G, Guilley H & Richards KE (1994). Artificial defective interfering RNAs derived from RNA 2 of beet necrotic yellow vein virus. *Arch Virol* **135,** 143-151.

Hehn A, Fritsch C, Richards KE, Guilley H & Jonard G (1997). Evidence for in vitro and in vivo autocatalytic processing of the primary translation product of beet necrotic yellow vein virus RNA 1 by a papain-like proteinase. *Arch Virol* **142,** 1051-1058.

Heidel GB, Rush CM, Kendall TL, Lommel SA & French RC (1997). Characteristics of beet soil-borne mosaic virus, a furo-like virus infecting sugar beet. *Plant Dis* **81,** 1070-1076.

Heijbroek W (1988). Dissemination of rhizomania by soil, beet seeds and stable manure. *Netherlands Journal of Plant Pathology* **94,** 9-15.

Heijbroek W (1989). The development of Rhizomania in two areas of the Netherlands and its effect on sugar-beet growth and quality. *Neth. J. Plant Pathol* **95,** 27-35.

Heijbroek W, Musters PMS & Schoone AHL (1999). Variation in pathogenicity and multiplication of beet necrotic yellow vein virus (BNYVV) in relation to the resistance of sugar-beet Cultivars. *European J. Plant Pathol.* **105,** 397-405.

Henry CM, Jones RAC & Coutts RHA (1986). Occurrence of a soil-borne virus of sugar beet in England. *plant pathology* **35,** 585-591.

Hugo SA, Henry CM & Harju V (1996). The role of alternative hosts of Polymyxa betae in transmission of beet necrotic yellow vein virus (BNYVV) in England. *plant pathology* **45,** 662-666.

Hull R (2002). *Plant Virology.* Academic press, London.

Hutchinson PJ, Henry CM & Coutts RH (1992). A comparison, using dsRNA analysis, between beet soil-borne virus and some other tubular viruses isolated from sugar beet. *J Gen Virol* **73 (Pt 5),** 1317-1320.

Jiang J-X, Zhang, J.-F., Che, S.-C., Yang, D.-J., Yu, J.-L., Cai, Z.-N. & Liu, Y. (1999). Transmission of beet black scorch virus by Olpidium brassicae. *J Jiangxi Agric Univ* **21,** 525-528.

Joersbo M & Brunstedt J (1990). Inoculation of sugar beet protoplasts with beet necrotic yellow vein virus particles by mild sonication. *J Virol Methods* **29,** 63-69.

Jones L, Keining T, Eamens A & Vaistij FE (2006). Virus-induced gene silencing of argonaute genes in Nicotiana benthamiana demonstrates that extensive systemic silencing requires Argonaute1-like and Argonaute4-like genes. *Plant Physiol* **141,** 598-606.

Jupin I, Guilley H, Richards KE & Jonard G (1992). Two proteins encoded by beet necrotic yellow vein virus RNA 3 influence symptom phenotype on leaves. *Embo J* **11,** 479-488.

Jupin I, Quillet L, Ziegler-Graff V, Guilley H, Richards K & Jonard G (1988). In vitro translation of natural and synthetic beet necrotic yellow vein virus RNA 1. *J Gen Virol* **69,** 2359-2367.

Jupin I, Tamada T & Richards K (1991). Pathogenesis of Beet necrotic yellow vein virus. *Semin. Virol* **2,** 121–129.

Kaufmann A, Koenig R & Rohloff H (1993). Influence of Beet soil-borne virus on mechanically inoculated sugar beet. *Plant Pathol* **42,** 413-417.

Keskin B (1964). [Polymyxa Betae N.Sp., a Parasite in the Roots of Beta Vulgaris Tournefort, Particularly During the Early Growth of the Sugar Beet.]. *Arch Mikrobiol* **49,** 348-374.

Kiguchi T, Saito M & Tamada T (1996). Nucleotide sequence analysis of RNA-5 of five isolates of beet necrotic yellow vein virus and the identity of a deletion mutant. *J Gen Virol* **77 (Pt 4),** 575-580.

Klein E, Link D, Schirmer A, Erhardt M & Gilmer D (2007). Sequence variation within Beet necrotic yellow vein virus p25 protein influences its oligomerization and isolate pathogenicity on Tetragonia expansa. *Virus Res* **126,** 53-61.

Koenig R (2000). Deletions in the KTER-encoding domain, which is needed for Polymyxa transmission, in manually transmitted isolates of Beet necrotic yellow vein benyvirus. *Arch Virol* **145,** 165-170.

Koenig R, Beier C, Commandeur U, Luth U, Kaufmann A & Luddecke P (1996). Beet soil-borne virus RNA 3--a further example of the heterogeneity of the gene content of furovirus genomes and of triple gene block-carrying RNAs. *Virology* **216,** 202-207.

Koenig R, Büttner G, Pferdmenges F, Herrenschwand W, Deml G & Varrelman M (2005). Distribution of various types of Beet necrotic yellow vein virus in Europe and abroad. In *Proceedings of the 6th Symposium of the International Working Group on Plant Viruses with Fungal Vectors (Abst.)*. ed. Rush Cm & Merz U, pp. 14., 2005. Bologna, Italy.

Koenig R, Commandeur U, Lesemann DE, Burgermeister W, Torrance L, Grassi G, Alric M, Kallerhoff J & Schots A (1990). Antigenic analysis of the coat protein of beet necrotic yellow vein virus by means of monocional antibodies *Journal of General Virology* **71,** 2229-2232.

Koenig R, Commandeur U, Loss S, Beier C, Kaufmann A & Lesemann DE (1997a). Beet soil-borne virus RNA 2: similarities and dissimilarities

to the coat protein gene-carrying RNAs of other furoviruses. *J Gen Virol* **78 (Pt 2),** 469-477.

Koenig R & Ehlers U (1989). Influence of the RNA composition of beet necrotic yellow vein furovirus isolates and of the sugarbeet cultivar on the translocation of the virus in mechanically inoculated sugarbeet roots. *EPPO Bulletin* **19,** 527-530.

Koenig R, Haeberle AM & Commandeur U (1997b). Detection and characterization of a distinct type of beet necrotic yellow vein virus RNA 5 in a sugarbeet growing area in Europe. *Arch Virol* **142,** 1499-1504.

Koenig R, Jarausch W, Li Y, Commandeur U, Burgermeister W, Gehrke M & Luddecke P (1991). Effect of recombinant beet necrotic yellow vein virus with different RNA compositions on mechanically inoculated sugarbeets. *J Gen Virol* **72 (Pt 9),** 2243-2246.

Koenig R, Kastirr U, Holtschulte B, Deml G & Varrelmann M (2008). Distribution of various types and P25 subtypes of Beet necrotic yellow vein virus in Germany and other European countries. *Arch Virol* **153,** 2139-2144.

Koenig R & Lennefors BL (2000). Molecular analyses of European A, B and P type sources of Beet necrotic yellow vein virus and detection of the rare P type in Kazakhstan. *Arch Virol* **145,** 1561-1570.

Koenig R, Lesemann DE & Burgermeister W (1984). Beet necrotic yellow vein virus: purification, preparation of antisera and detection by means of ELISA, immunosorbent electron microscopy and electro-blot immunoassay. *Phytopathologische Zeitschrift* **111,** 244–252.

Koenig R & Loss S (1997). Beet soil-borne virus RNA 1: genetic analysis enabled by a starting sequence generated with primers to highly conserved helicase-encoding domains. *J Gen Virol* **78 (Pt 12),** 3161-3165.

Koenig R, Luddecke P & Haeberle AM (1995). Detection of beet necrotic yellow vein virus strains, variants and mixed infections by examining single-strand conformation polymorphisms of immunocapture RT-PCR products. *J Gen Virol* **76 (Pt 8),** 2051-2055.

Koenig R, Pleij CW, Beier C & Commandeur U (1998). Genome properties of beet virus Q, a new furo-like virus from sugarbeet, determined from unpurified virus. *J Gen Virol* **79 (Pt 8),** 2027-2036.

Koenig R, Pleij CW & Buttner G (2000). Structure and variability of the 3' end of RNA 3 of Beet soil-borne pomovirus--a virus with uncertain pathogenic effects. *Arch Virol* **145,** 1173-1181.

Koenig R & Valizadeh J (2008). Molecular and serological characterization of an Iranian isolate of Beet black scorch virus. *Arch Virol* **153,** 1397-1400.

Koonin EV & Dolja VV (1993). Evolution and taxonomy of positive-strand RNA viruses: implications of comparative analysis of amino acid sequences. *Crit Rev Biochem Mol Biol* **28,** 375-430.

Koonin EV, Gorbalenya AE, Purdy MA, Rozanov MN, Reyes GR & Bradley DW (1992). Computer-assisted assignment of functional domains in the nonstructural polyprotein of hepatitis E virus: delineation of an additional group of positive-strand RNA plant and animal viruses. *Proc Natl Acad Sci U S A* **89,** 8259-8263.

Krabovic J & Kralova V (1996). Relations among rhizomania disease, root content of potassium, sodium, α-amino nitrogen and crop yield of sugar beet. *Journal of Plant Disease and Protection* **103,** 561-570.

Kruse M, Koenig R, Hoffmann A, Kaufmann A, Commandeur U, Solovyev AG, Savenkov I & Burgermeister W (1994). Restriction fragment length polymorphism analysis of reverse transcription-PCR products reveals the existence of two major strain groups of beet necrotic yellow vein virus. *J Gen Virol* **75 (Pt 8),** 1835-1842.

Kuroda T, Natsuaki T, Wang WQ & Okuda S (1997). Formation of multimers of cucumber mosaic virus satellite RNA. *J. Gen. Virol.* **78,** 941–946.

Kutluk Yilmaz ND, Meunier A, Schmit JF, Stas A & Bragard C (2007). Partial nucleotide sequence analysis of Turkish isolates of Beet necrotic yellow vein virus (BNYVV) RNA-3. *Plant Pathol* **56,** 311-316.

Larson RL, Wintermantel WM, Hill A, Fortis L & Nunez A (2008). Proteome changes in sugar beet in response to Beet necrotic yellow vein virus. *Physiol. Mol. Plant Pathol* **72,** 67–72.

Lauber E, Bleykasten-Grosshans C, Erhardt M, Bouzoubaa S, Jonard G, Richards KE & Guilley H (1998). Cell-to-cell movement of beet necrotic yellow vein virus: I. Heterologous complementation experiments provide evidence for specific interactions among the triple gene block proteins. *Mol Plant Microbe Interact* **11,** 618-625.

Lauber E, Guilley H, Richards K, Jonard G & Gilmer D (1997). Conformation of the 3'-end of beet necrotic yellow vein benevirus RNA 3 analysed by chemical and enzymatic probing and mutagenesis. *Nucleic Acids Res* **25,** 4723-4729.

Lee L, Telford EB, Batten JS, Scholthof KB & Rush CM (2001). Complete nucleotide sequence and genome organization of Beet soilborne mosaic virus, a proposed member of the genus Benyvirus. *Arch Virol* **146,** 2443-2453.

Legrève A, Delfosse P & Maraite H (2002). Phylogenetic analysis of Polymyxa species based on nuclear 5.8S and internal transcribed spacers ribosomal DNA sequences. *Mycological Research* **106,** 138–147.

Legrève A & Maraite H (1992). Influence du calcium et du pH sur le potenciel du sol en *polymyxa betae* KZSKIN et son expression en champ. In *Compets rendus du cinquante-cinquième congrès d'hiver de l'institut de Recherches Betteravières (IIRB), Bruxelles.*

Lemaire O, Beuve M, Weber C, Schirmer A, Link D, Meunier A, Bragard C & Gilmer D (2003). Etiology and molecular epidemiology of a severe rhizomania disease occurring in confined locations in Europe: hypothesis for the implication of the RNA-3 and/or-5 of Beet necrotic yellow vein virus (BNYVV)-P pathotype. In *Proceedings of the 1st joint IIRBASSBT Congress*, pp. 303-318, San Antonio.

Lemaire O, Merdinoglu D, Valentin P, Putz C, Ziegler-Graff V, Guilley H, Jonard G & Richards K (1988). Effect of beet necrotic yellow vein virus RNA composition on transmission by Polymyxa betae. *Virology* **162,** 232-235.

Lennefors B-L, Lindsten K & Koenig R (2000). First record of A and B type Beet necrotic yellow vein virus in sugar beets in Sweden. *Eur. J. Plant Pathol.* **106,** 199–201.

Lennefors BL (2007). Molecular breeding for resistance to rhizomania in sugar beets, vol. phD, pp. 106. Swedish University of Agricultural Sciences, Uppsala, Sweden.

Lesemann DE, Koenig R, Lindsten K & Henry C (1989). Serotypes of beet soil-borne furovirus from FRG and Sweden. *Buttetin OEPP/EPPO Bulletin***,** 539-540.

Li D, Yu J, Han C, Lui T, Quin S, Lui Y & Koenig R (1999). Detection and nucleotide sequence analysis of Beet necrotic yellow vein virus RNA 5 isolated from China. *Chinese Journal of Biotechnology* **15,** 461-465.

Li J, Li M, Li C, Gao Y, Li D, Han C & Yu J (2008a). Effects on the local symptoms of subgenomic RNAs expressions and their translational products of Tobacco necrosis virus A Chinese isolate. *Chinese Science Bulletin* **53,** 1682-1690.

Li M, Liu T, Wang B, Han C, Li D & Yu J (2008b). Phylogenetic analysis of Beet necrotic yellow vein virus isolates from China. *Virus Genes* **36,** 429-432.

Li Y, Wei C, Tien P, Pan N & Chen Z (1996). Immunodetection of beet necrotic yellow vein virus RNA3-encoded protein in different host plants and tissues. *Acta Virol* **40,** 67-72.

Lindsten K (1989). Investigations concerning soil-bome viruses in sugarbect in Sweden. *Bullelin OEPH EPPO Bulletin* **19,** 531-557.

Link D, Schmidlin L, Schirmer A, Klein E, Erhardt M, Geldreich A, Lemaire O & Gilmer D (2005). Functional characterization of the Beet necrotic yellow vein virus RNA-5-encoded p26 protein: evidence for structural pathogenicity determinants. *J Gen Virol* **86,** 2115-2125.

Littlefield L & Whallon J (1999). Comparative anatomy, taxonomy and biology of Polymyxa betae and Polymyxa graminis: an overview. In *Proc. Symp. Int. Work. Group on Plant Viruses with Fungal Vectors, 4th.*, pp. 93–96. Am. Soc. Sugar Beet Technol, Denver.

Liu H, Sears L & Lewellen RT (2005). Occurrence of Resistance-Breaking Beet necrotic yellow vein virus of sugar beet. *plant disease* **89,** 464-468.

Liu HY & Lewellen RT (2007). Distribution and molecular characterization of resistance-breaking isolates of Beet necrotic yellow vein virus in the United States. *Plant Dis* **91,** 847–851.

Liu J & Xian H (1995). Preliminary report on Beet black scorch virus. *China Sugar Beet* **3,** 30-31.

Lommel SA, Martelli, G. P., Rubino, L. & Russo, M. (2005). Tombusviridae. In *Virus Taxonomy. Eighth Report of the International Committee on Taxonomy of Viruses*. ed. C. M. Fauquet Mam, J. Maniloff, U. Desselberger & L. A. Ball, pp. 906–936. Elsevier/Academic Press, Lodon.

Lubicz JV, Rush CM, Payton M & Colberg T (2007). Beet necrotic yellow vein virus accumulates inside resting spores and zoosporangia of its vector Polymyxa betae BNYVV infects P. betae. *Virol J* **4,** 37.

Mandahar C (2006). *Multiplication of RNA plant viruses*. Springer, Dordrecht.

McGrann GR, Grimmer MK, Mutasa-Gottgens ES & Stevens M (2009). Progress towards the understanding and control of sugar beet rhizomania disease. *Mol Plant Pathol* **10,** 129-141.

Mehrvar M, Valizadeh J, Koenig R & Bragard CG (2009). Iranian beet necrotic yellow vein virus (BNYVV): pronounced diversity of the p25 coding region in A-type BNYVV and identification of P-type BNYVV lacking a fifth RNA species. *Arch Virol* **154,** 501-506.

Merdinoglu D, Valentin P, Geyl L, Merdinoglu- Wiedemann S, Lemaire O & Putz C (1993). Amelioration genetique de la betterave pour la resistance a la rhizomanie. *Comptes Rendus de t'Academie d'Agricutture Franfaise* **79,** 85-98.

Meunier A (2005). Diversity and characterization of Beet necrotic yellow vein virus RNA-2 and RNA-3. In *plant pathology*, vol. PhD, pp. 122. UCL, Louvain-la-Neuve, Belgium.

Meunier A, Schmit JF & Bragard C (2005). Comparison of the beet necrotic yellow vein virus P75 nucleotide sequences of Belgian type A and type B sources. *Virus Res* **108,** 15-22.

Meunier A, Schmit JF, Stas A, Kutluk N & Bragard C (2003). Multiplex reverse transcription-PCR for simultaneous detection of beet necrotic yellow vein virus, Beet soilborne virus, and Beet virus Q and their vector Polymyxa betae KESKIN on sugar beet. *Appl Environ Microbiol* **69,** 2356-2360.

Meunier A, Schmit JF, Stas A, Marlier A, Wauters A, Steyer S & Bragard C (2000). The status of rhizomania in Belgium. *Parasitica* **56,** 85-95

Miller WA & Koev G (2000). Synthesis of subgenomic RNAs by positive-strand RNA viruses. *Virology* **273,** 1-8.

Miyanishi M, Kusume T, Saito M & Tamada T (1999). Evidence for three groups of sequence variants of beet necrotic yellow vein virus RNA 5. *Arch Virol* **144,** 879-892.

Moissiard G & Voinnet O (2004). Viral suppression of RNA silencing in plant virues. *Mol Plant Pathol* **5,** 71-82.

Molnar A, Havelda Z, Dalmay T, Szutorisz H & Burgyan J (1997). Complete nucleotide sequence of tobacco necrosis virus strain DH and genes required for RNA replication and virus movement. *J Gen Virol* **78 (Pt 6),** 1235-1239.

Morris J, Clover GR, Harju VA, Hugo SA & Henry CM (2001). Development of a highly sensitive nested RT-PCR method for Beet necrotic yellow vein virus detection. *J Virol Methods* **95,** 163-169.

Murant A & Mayo M (1982). Satellites of plant viruses. *Annu Rev Phytopathol* **20,** 49-70.

Niesbach-Klosgen U, Guilley H, Jonard G & Richards K (1990). Immunodetection in vivo of beet necrotic yellow vein virus-encoded proteins. *Virology* **178,** 52-61.

Oda Y, Saeki K, Takahashi Y, Maeda T, Naitow H, Tsukihara T & Fukuyama K (2000). Crystal structure of tobacco necrosis virus at 2.25 A resolution. *J Mol Biol* **300,** 153-169.

Pferdmenges F (2007). Occurrence, spread and pathogenicity of different Beet necrotic yellow vein virus (BNYVV) isolates, vol. PhD, pp. 110. University of Gotteningen, Gotteningen.

Piccinni G & Rush CM (2000). Determination of Optimum Irrigation Regime and Water Use Efficiency of Sugar Beet Grown in Pathogen Infested Soil. *Plant Dis* **84,** 1067-1072.

Prillwitz H & Schlösser E (1992). Beet soil-borne virus: occurrence, symptoms and effect on plant development. *Mededelingen van de Faculteit Landbouwwetenschapen, Rijksuniversiteit Gent* **57**.

Putz C (1977). Composition and Structure of Beet Necrotic Yellow Vein Virus *Journal of General Virology* **35,** 397-401.

Qu F, Ren T & Morris TJ (2003). The coat protein of turnip crinkle virus suppresses posttranscriptional gene silencing at an early initiation step. *J Virol* **77,** 511-522.

Rahim MD, Andika IB, Han C, Kondo H & Tamada T (2007). RNA4-encoded p31 of beet necrotic yellow vein virus is involved in efficient vector transmission, symptom severity and silencing suppression in roots. *J Gen Virol* **88,** 1611-1619.

Ratti C, Clover GR, Autonell CR, Harju VA & Henry CM (2005). A multiplex RT-PCR assay capable of distinguishing beet necrotic yellow vein virus types A and B. *J Virol Methods* **124,** 41-47.

Ratti C, Hleibieh K, Bianchi L, Schirmer A, Autonell CR & Gilmer D (2009). Beet soil-borne mosaic virus RNA-3 is replicated and encapsidated in the presence of BNYVV RNA-1 and -2 and allows long distance movement in Beta macrocarpa. *Virology* **385,** 392-399.

Ratti C, Resca R, Lanzoni C & Rubies Autonell C (2006). Amino acid changes in the 25 protein of Beet necrotic yellow vein virus. In *Proceedings of the 6th symposium of the international working group on plant viruses with fungal vectors*. ed. Rush Cm & Merz U, pp. 53, Bologna.

Richards K & Tamada T (1992). Mapping functions on the multipartite genome of beet necrotic yellow vein virus. *Annu. Rev. Phytopathol.* **30,** 291-313.

Rochon D, Kakani K, Robbins M & Reade R (2004). Molecular aspects of plant virus transmission by olpidium and plasmodiophorid vectors. *Annu Rev Phytopathol* **42,** 211-241.

Roossinck MJ, Sleat D & Palukaitis P (1992). Satellite RNAs of plant viruses: structures and biological effects. *Microbiol Rev* **56,** 265-279.

Rush CM (2003). Ecology and epidemiology of benyviruses and plasmodiophorid vectors. *Annu Rev Phytopathol* **41,** 567-592.

Rush CM & Heidel GB (1995). Furovirus diseases of sugar beets in the United States. *Plant Dis.* **79,** 868-875.

Rush CM, Liu HY, Lewellen.R.T & Acosta-Leal R (2006). The continuing saga of rhizomania of sugar beets in the United States. *Plant Dis* **90,** 4-15.

Rush CM, Scholthof KBG, Manohar SK & Heidel GB (1996). Similarities between beet soilborne mosaic virus and beet necrotic yellow vein virus RNA2 nucleotide sequence and genomic organization. In *Proc. Third Symp. Intl. Working Group Plant Viruses with Fungal Vectors*, pp. 29-32, Dundee, Scotland.

Russo M, Martelli GP & Di Franco A (1981). he fine structure of local lesions of beet necrotic yellow vein virus in Chenopodium amaranticolor. *Physiological Plant Pathology* **19,** 237-242.

Rysanek P, Stocky G, Haeberle AM & Putz C (1992). Immunogold labelling of Beet necrotic yellow vein virus particles inside its fungal vector, Polymyxa betae K. *Agronomie.* **12,** 651-659.

Saito M, Kiguchi T, Kusume T & Tamada T (1996). Complete nucleotide sequence of the Japanese isolate S of beet necrotic yellow vein virus RNA and comparison with European isolates. *Arch Virol* **141,** 2163-2175.

Saitou N & Nei M (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4,** 406-425.

Schirmer A, Link D, Cognat V, Moury B, Beuve M, Meunier A, Bragard C, Gilmer D & Lemaire O (2005). Phylogenetic analysis of isolates of Beet necrotic yellow vein virus collected worldwide. *J Gen Virol* **86,** 2897-2911.

Schmitt C, Balmori E, Jonard G, Richards KE & Guilley H (1992). In vitro mutagenesis of biologically active transcripts of beet necrotic yellow vein virus RNA 2: evidence that a domain of the 75-kDa readthrough protein is important for efficient virus assembly. *Proc Natl Acad Sci U S A* **89,** 5715-5719.

Shen R & Miller WA (2004). The 3' untranslated region of tobacco necrosis virus RNA contains a barley yellow dwarf virus-like cap-independent translation element. *J Virol* **78,** 4655-4664.

Shen R & Miller WA (2007). Structures required for poly(A) tail-independent translation overlap with, but are distinct from, cap-independent translation and RNA replication signals at the 3' end of Tobacco necrosis virus RNA. *Virology* **358,** 448-458.

Sherwood JL & Rush CM (1999). In *Proc. Symp. Int. Work. Group on Plant Viruses with Fungal Vectors, 4th.* , pp. 142. Am. Soc. Sugar Beet Technol, Denver.

Simon AE, Roossinck MJ & Havelda Z (2004). Plant virus satellite and defective interfering RNAs: new paradigms for a new century. *Annu Rev Phytopathol* **42,** 415-437.

Sohi HH & Maleki M (2004). Evidence for presence of types A and B of beet necrotic yellow vein virus (BNYVV) in Iran. *Virus Genes* **29,** 353-358.

Strausbaugh CA, Rearick E & Gallian JJ (2008). Influence of Beet necrotic yellow vein virus on sugar beet storability. *Plant Dis* **92,** 581–587.

Sun Q, Choi GH & Nuss DL (2009). A single Argonaute gene is required for induction of RNA silencing antiviral defense and promotes viral RNA recombination. *Proc Natl Acad Sci U S A* **106,** 17927-17932.

Szittya G & Burgyan J (2001). Cymbidium ringspot tombusvirus coat protein coding sequence acts as an avirulent RNA. *J Virol* **75,** 2411-2420.

Tamada T (1975). Beet necrotic yellow vein virus. In *CMI/AAB Descriptions of Plant Viruses No. 144*, Wellesbourne, UK.

Tamada T & Abe H (1989). Evidence that beet necrotic yellow vein virus RNA-4 is essential for transmission by the fungus Polymyxa betae *J Gen Virol* **70,** 3391-3393.

Tamada T & Baba T (1973). Beet necrotic yellow vein virus from rhizomania-affected sugar beet in Japan. *Ann. Phytopathol. Soc. Japan* **39,** 325-332.

Tamada T & Kusume T (1991). Evidence that the 75K readthrough protein of beet necrotic yellow vein virus RNA-2 is essential for transmission by the fungus Polymyxa betae *Journal of General Virology* **72,** 1497-1504.

Tamada T, Kusume T, Uchino H, Kigushi T & Saito M (1996a). Evidence that beet necrotic yellow vein virus RNA-5 is involved in symptom development of sugar beet root. In *Proceedings of the Third Symposium of the International Working Group on Plant Viruses*

with Fungal Vectors. ed. Sherwood Jl & Rush Cm, pp. 49–52, Denver: American Society of Sugar Beet Technologists.

Tamada T, Miyanishi M, Kondo H, Chiba H & Han G (2002). Pathogenicity and molecular variability of Beet necrotic yellow vein virus isolates from Europe, Japan, China and The United States. In *Proceedings of the 5th Symposium of the International Working Group on Plant Viruses with Fungal Vectors*. ed. Rush Cm & Merz U, pp. 13–16, Zurich, Switzerland.

Tamada T, Schmitt C, Saito M, Guilley H, Richards K & Jonard G (1996b). High resolution analysis of the readthrough domain of beet necrotic yellow vein virus readthrough protein: a KTER motif is important for efficient transmission of the virus by Polymyxa betae. *J Gen Virol* **77 (Pt 7),** 1359-1367.

Tamada T, Shirako Y, Abe H, Saito M, Kiguchi T & Harada T (1989). Production and pathogenicity of isolates of beet necrotic yellow vein virus with different numbers of RNA components. *Journal of General Virology* **70,** 3399–3409.

Tamada T, Uchino H, Kusume T & Saito M (1999). RNA 3 deletion mutants of beet necrotic yellow vein virus do not cause rhizomania disease in sugar beets. *Phytopathology* **89,** 1000-1006.

Tamura K, Dudley J, Nei M & Kumar S (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* **24,** 1596-1599.

Thomas CL, Leh V, Lederer C & Maule AJ (2003). Turnip crinkle virus coat protein mediates suppression of RNA silencing in Nicotiana benthamiana. *Virology* **306,** 33-41.

Torrance L (2008). Pomovirus. *Elsevier***,** 1-5.

Torrance L, Pead MT & Buxton G (1988). characteristics of monoclonal antibodies against beet necrotic yellow vein virus. *Annals of Applied Biology* **113,** 519-530.

Tuitert G (1993). Horizontal spread of Beet necrotic yellow vein virus in soil. *Neth. J. Plant Pathol* **99,** 85–96.

Tuitert G & Hofmeester Y (1992). Epidemiology of beet necrotic yellow vein virus in sugar beet at dierent initial inoculum levels in the

presence or absence of irrigation. *Netherlands Journal of Plant Pathology* **98,** 343-360.

Tuitert G & Hofmeester Y (1994). Epidemiology of Beet necrotic yellow vein virus in sugar beet at different initial inoculum levels in the presence and absence of irrigation: disease incidence yield and quality. *Europ.J. Plant Pathol* **100,** 19-53.

Uhde K, Kerschbaumer RJ, Koenig R, Hirschl S, Lemaire O, Boonham N, Roake W & Himmler G (2000). Improved detection of Beet necrotic yellow vein virus in a DAS ELISA by means of antibody single chain fragments (scFv) which were selected to protease-stable epitopes from phage display libraries. *Arch Virol* **145,** 179-185.

Valentin C, Dunoyer P, Vetter G, Schalk C, Dietrich A & Bouzoubaa S (2005). Molecular basis for mitochondrial localization of viral particles during beet necrotic yellow vein virus infection. *J Virol* **79,** 9991-10002.

Van Regenmortel MHV, Fauquet CM, Bishop DHL, Carstens E, Estes MK, Lemon S, McGeoch D, Wickner RB, Mayo MA, Pringle CR & Maniloff J (2000). *Virus taxonomy. Seventh report of the International Committee on Taxonomy of Viruses*. Academic Press, New York, N.Y.

Verhoyen M, Van Den Bossche M & Van Steyvoort M (1987). Identification de nouveaux virus de la betterave en Belgique. *Revue Agric. Brux.* **40,** 1463.

Vetter G, Hily JM, Klein E, Schmidlin L, Haas M, Merkle T & Gilmer D (2004). Nucleo-cytoplasmic shuttling of the beet necrotic yellow vein virus RNA-3-encoded p25 protein. *J Gen Virol* **85,** 2459-2469.

Wang B, Li M, Han C, Li D & Yu J (2008). Complete Genome Sequences of Two Chinese Beet soil-borne virus Isolates Provide Evidence that the Genome is Highly Conserved. *J. Phytopathol.* **156,** 487 - 488.

Wang J & Simon AE (2000). 3'-End stem-loops of the subviral RNAs associated with turnip crinkle virus are involved in symptom modulation and coat protein binding. *J Virol* **74,** 6528-6537.

Wang MB, Bian XY, Wu LM, Liu LX, Smith NA, Isenegger D, Wu RM, Masuta C, Vance VB, Watson JM, Rezaian A, Dennis ES & Waterhouse PM (2004). On the role of RNA silencing in the

pathogenicity and evolution of viroids and viral satellites. *Proc Natl Acad Sci U S A* **101,** 3275-3280.

Ward L, Fenn M & Henry C (2004). Development of a direct detection method for Polymyxa sp. in soil. In *Proc. Symp. Int. Work. Group on Plant Viruses with Fungal Vectors, 5th*. ed. Rush Cm & Merz U, pp. 111–114. Am. Soc.Sugar Beet Technol, Denver.

Ward L, Koenig R, Budge G, Garrido C, McGrath C, Stubbley H & Boonham N (2007). Occurrence of two different types of RNA-5-containing beet necrotic yellow vein virus in the UK. *Arch Virol* **152,** 59-73.

Wauters A, Legrand G & Tits M (1997). Reconnaitre la rhizomanie sur-le-champ. Publication rèalisèe dans le cadre du Centre Agricole Betterave-Chicorèe (CABC-LBCC). Institut Royal belge pour l'amélioration de la betterave (IIRBAB/KBIVB). ed. Institut Royal Belge Pour L'amélioration De La Betterave (Iirbab/Kbivb) T, Belgium. Ministères Des Classes Moyennes Et De L'agriculture, Service Recherrche Et Développement (Dg Vi), pp. 11-14.

Weiland JJ & Edwards MC (1994). Evidence that the alpha a gene of barley stripe mosaic virus encodes determinants of pathogenicity to oat (Avena sativa). *Virology* **201,** 116-126.

Weiland JJ, Van Winkle D, Edwards MC, Larson RL, Shelver WL, Freeman TP & Liu HY (2007). Characterization of a U.S. Isolate of Beet black scorch virus. *Phytopathology* **97,** 1245-1254.

White KA & Nagy PD (2004). Advances in the molecular biology of tombusviruses: gene expression, genome replication, and recombination. *Prog Nucleic Acid Res Mol Biol* **78,** 187-226.

Wisler GC, Lewellen RT, Sears JL, Liu HY & Duffus JE (1999). Specificity of TAS-ELISA for beet necrotic yellow vein virus and its application for determining rhizomania resistance in field grown sugar beet. *Plant Dis.* **83,** 864-870.

Wisler GC, Lewellen RT, Sears JL, Wasson JW, Liu HY & Wintermantel WM (2003). Interactions between Beet necrotic yellow vein virus and Beet soilborne mosaic virus in sugar beet. *Plant Dis* **87,** 1170–1175.

Yuan X, Cao Y, Xi D, Guo L, Han C, Li D, Zhai Y & Yu J (2006). Analysis of the subgenomic RNAs and the small open reading frames of Beet black scorch virus. *J Gen Virol* **87,** 3077-3086.

Zhang K-W, Wang, S.-L., Cai, Z.-N. & Zhang, H.-S. (1996). Preliminary study on beet black scorch disease in Ningxia Province. *China Sugar Beet* **2,** 3-8.

Zhao Y, DelGrosso L, Yigit E, Dempsey DA, Klessig DF & Wobbe KK (2000). The amino terminus of the coat protein of Turnip crinkle virus is the AVR factor recognized by resistant arabidopsis. *Mol Plant Microbe Interact* **13,** 1015-1018.

Zuker M (2003). Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* **31,** 3406-3415.

Chapter X. Annexes

X.1. Full-length infectious clone of *beet soil-borne virus* indicates the dispensability of the RNA-2 for virus survival *in planta* and symptom expression on *Chenopodium quinoa* leaves

Journal of General Virology (2009), 90, 3051–3056 DOI 10.1099/vir.0.014548-0

Short Communication

A full-length infectious clone of beet soil-borne virus indicates the dispensability of the RNA-2 for virus survival *in planta* and symptom expression on *Chenopodium quinoa* leaves

François Crutzen,[1] Mohsen Mehrvar,[1,2] David Gilmer[3] and Claude Bragard[1]

Correspondence
François Crutzen
francois.crutzen@uclouvain.be

[1]Université catholique de Louvain, unité de phytopathologie, Croix du Sud 2 bte 3, B-1348 Louvain-la-Neuve, Belgium

[2]Department of Plant Protection, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran

[3]Institut de Biologie Moléculaire des Plantes, Laboratoire propre du CNRS (UPR 2357) conventionné avec l'Université de Strasbourg, 12 rue du Général Zimmer, 67084 Strasbourg, France

Received 23 June 2009
Accepted 1 September 2009

For a better understanding of the functionality and pathogenicity of beet soil-borne virus (BSBV), full-length cDNA clones have been constructed for the three genomic RNAs. With the aim of assessing their effectiveness and relative contribution to the virus housekeeping functions, transcripts were inoculated on *Chenopodium quinoa* and *Beta macrocarpa* leaves using five genome combinations. Both RNAs-1 (putative replicase) and -3 (putative movement proteins) proved to be essential for virus replication *in planta* and symptom production on *C. quinoa*, whereas RNA-2 (putative coat protein, CP, and a read-through domain, RT) was not. No symptoms were recorded on *B. macrocarpa*, but viral RNAs were detected. In both host plants, the 19 kDa CP was detected by Western blotting as well as a 115 kDa protein corresponding to the CP–RT.

Beet soil-borne virus (BSBV) is a pomovirus transmitted to Chenopodiaceae by the protist *Polymyxa betae* (Ivanović *et al.*, 1983), which is also the vector of the aetiological agent of the rhizomania syndrome of sugar beet, beet necrotic yellow vein virus (BNYVV) (Tamada & Baba, 1973). Originally reported in Italy (Canova, 1959), rhizomania disease is now widespread in most countries where sugar beet is grown (McGrann *et al.*, 2009) and BSBV is often found in beet infected with BNYVV (Meunier *et al.*, 2003). However, the pathogenicity of BSBV and its contribution to the rhizomania syndrome remain unclear, with opinions still divided on this (Prillwitz & Schlösser, 1992; Kaufmann *et al.*, 1993; Lindsten, 1993; Rush & Heidel, 1995).

The BSBV genome consists of three single-stranded RNAs of positive polarity, packaged into rod-shaped particles (Koenig *et al.*, 1996, 1997; Koenig & Loss, 1997). RNA-1 (5.8 kb) encodes the putative viral replicase. The 19 kDa coat protein (CP) and a putative 85 kDa read-through (RT) domain are encoded by RNA-2 (3.5 kb). RNA-3 (3.0 kb) comprises three open reading frames (ORFs) encoding three putative proteins (48, 13 and 22 kDa) thought to be responsible for the viral cell-to-cell movement, resembling the well-known triple gene block proteins (TGBs) (Fig. 1).

In this study, the contribution of each RNA component to virus survival and symptom expression was investigated through the use of full-length cDNA clones on two host plants, *Chenopodium quinoa* and *Beta macrocarpa*. This last plant species was preferred to the natural host *Beta vulgaris* with a view of developing the basis for further molecular analysis of viral systemicity, following the example of previous studies on BNYVV (Lauber *et al.*, 1998).

Full-length cDNA sequences were generated from an Iranian BSBV isolate (Nyshabour, Khorasan Razavi Province), which was trapped from infested soil in roots of *B. vulgaris*. After total RNA extraction from sugar beet roots using the SV total RNA isolation kit (Promega), the three genomic RNAs were reverse transcribed and amplified by PCR (RT-PCR) using expand reverse transcriptase and the expand long template PCR System (Roche). Primers matching the extremities of the three viral RNAs were designed following the only references known to date (Koenig *et al.*, 1996, 1997; Koenig & Loss, 1997). Forward primers were flanked with a T7 promoter sequence (TAATACGACTCACTATAG) (Fig. 1). Ampli-

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are FN386612, FN386613 and FN386614.

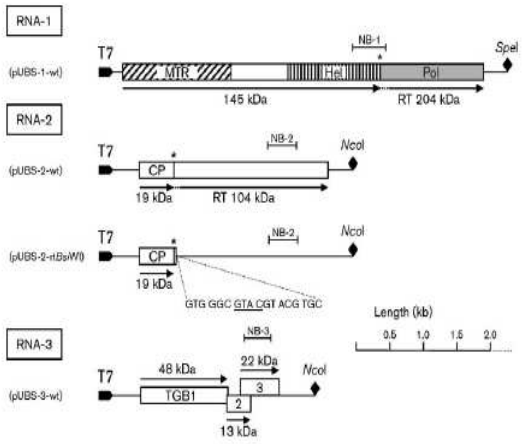


Fig. 1. Schematic representation of the BSBV tripartite genome: RNA-1 encodes the putative replicase with conserved motifs methyl-transferase (MTR), helicase (Hel) and RNA-dependent RNA polymerase (Pol) translated as an RT domain; RNA-2 encodes the putative CP and an RT domain; RNA-3 encodes the putative TGBs-1, -2 and -3. The asterisks (*) represent the RT stop codons. The molecular mass of the putative proteins is given beside each arrow beneath or above the corresponding ORF. The full-length cDNA clones (pUBS-1-wt, -2-wt, -2-rt*Bsi*WI, -3-wt; pUC19 backbone) are under the control of a T7 promoter and possess a unique restriction site (◆) for linearization and run-off transcription. pUBS-2-rt*Bsi*WI clone has a frameshift at the beginning of the CP–RT domain coding sequence as a result of the 4 nt insertion (underlined). The probes used for Northern blot detection of the three viral RNAs are positioned above the corresponding full-length clone (NB-1, -2 and -3).

cons were gel purified using QIAquick gel extraction kit (Qiagen) and inserted into the pGEM-T vector (Promega). Ligation products were transferred into the *Escherichia coli* strain JM109. Full-length cDNA clones were then sub-cloned into pUC19 and maintained in the *E. coli* strain DH5-α, to obtain the final constructs pUBS-1-wt (RNA-1), pUBS-2-wt (RNA-2) and pUBS-3-wt (RNA-3) (Fig. 1). A full-length cDNA clone of RNA-2 containing a frame shift mutation in the coding sequence for the putative CP–RT domain (done pUBS-2-rt*Bsi*WI, Fig. 1) was obtained by filling a *Bsi*WI digestion (target site position 907–912) with DNA polymerase I Klenow fragment (Promega) followed by self-ligation with T4 DNA ligase (Roche).

The four full-length cDNA constructs in pUC19 were linearized with *Spe*I or *Nco*I (Fig. 1) before run-off transcription with the RiboMAX large-scale RNA production system - SP6 and T7 (Promega) in the presence of Cap analogue $m^7G(5')ppp(5')G$ (New England Biolabs). The transcripts were mechanically inoculated in a 50 mM KH_2PO_4 (pH 4.2) and 0.04 % bentonite buffer on both *C. quinoa* and *B. macrocarpa* leaves. *In vitro* transcript combinations of RNA-1 (1), -2 (2), -2 carrying a frame shift mutation in the RT domain coding sequence (2*) and -3 (3) were tested as follows: 1+2+3, 1+2*+3, 1+2, 1+3 and 2+3. Inoculation buffer alone was applied for the negative control. *C. quinoa* and *B. macrocarpa* were exposed to light for 16 h per day at 15–20 °C and 20–25 °C for night and day, respectively. The experiment was carried out on one *C. quinoa* plant (three leaves inoculated) and two *B. macrocarpa* plants (two leaves inoculated per plant) for each condition and repeated twice to ensure reproducibility.

Symptomatic lesions appeared on all the *C. quinoa* leaves inoculated with BSBV full-length transcripts of RNAs 1+2+3, 1+2*+3 or 1+3 at 3 days post-inoculation (p.i.). Small necrotic spots, necrotic ringspots (data not shown) and chlorosis were clearly developed 7 days p.i. (Fig. 2). However, the infection of the *B. macrocarpa* plants with the same three combinations of BSBV full-length transcripts did not produce any symptoms on the inoculated leaves (data not shown). These observations indicated that the presence of the BSBV RNA-2 was therefore not necessary for symptom expression, which occurred only on *C. quinoa*.

C. quinoa- and *B. macrocarpa*-inoculated leaves were harvested at 7 and 10–17 days p.i., respectively. The RNAs were extracted from the whole leaves using the polysomes extraction protocol (Jupin *et al.*, 1990) followed by phenol extraction and ethanol precipitation of the

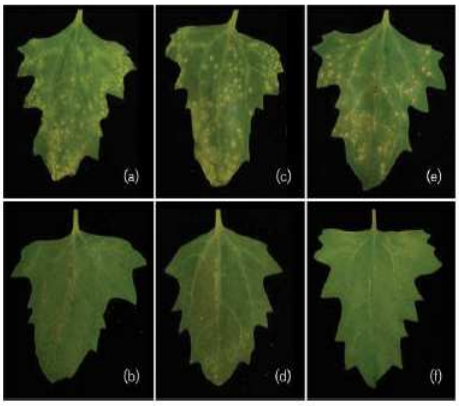


Fig. 2. *C. quinoa* leaves 7 days after inoculation with BSBV full-length transcript mixes 1+2*+3 (a), 1+2 (b), 1+2+3 (c), 2+3 (d) and 1+3 (e) and a negative control (f). Only genome arrangements 1+2*+3 (a), 1+2+3 (c) and 1+3 (e) provoked the appearance of symptoms on the inoculated leaves, with necrotic spots and ringspots (data not shown) and slight yellow chlorosis.

RNAs. The viral RNAs were detected by Northern blot with random DNA probes synthesized with the Prime-a-Gene Labelling System (Promega) and labelled with [α-^{32}P]dCTP (1.14×10^{14} Bq mmol^{-1}; Perkin Elmer). The probes matched nucleotide positions 3510–4015 (NB-1), 2281–2711 (NB-2) and 1930–2330 (NB-3) of RNAs-1, -2 and -3, respectively (Fig. 1).

Genomic combinations 1+2*+3, 1+2+3 and 1+3 resulted in corresponding viral RNA amplification on both host plants (Fig. 3a, b, lanes 1, 2 and 4), whereas 1+2 and 2+3 did not (Fig. 3a, b, lanes 3 and 5). The BSBV RNAs -1 and -3 full-length transcripts were therefore required for the efficient viral replication *in planta*, in contrast with BSBV RNA-2 that appeared dispensable for the viral RNA replication. However, even if no viral RNAs were detected in plants inoculated with the combinations 1+2 and 2+3 (Fig. 3a, b, lanes 3 and 5), a potential undetected infection cannot be ruled out for the mix 1+2. Lacking the putative cell-to-cell movement proteins encoded by RNA-3, the RNAs-1 and -2 might indeed replicate in the first inoculated cells but couldn't spread to the neighbouring ones (Hull, 2009). This will be further characterized using protoplast inoculation.

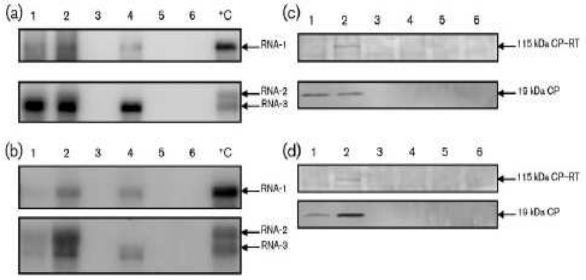


Fig. 3. Northern (a, b) and Western (c, d) blot analysis for the detection of BSBV RNAs and structural proteins in *C. quinoa* (a, c) and *B. macrocarpa* (b, d) leaves, 7 and 10–17 days p.i., respectively. Plant leaves were either inoculated with one of the five genome arrangements of BSBV full-length transcripts tested [combinations 1+2*+3 (lane 1), 1+2+3 (2), 1+2 (3), 1+3 (4) and 2+3 (5)] or mock-inoculated (6). The three BSBV genomic RNAs were detected in total RNA extracts using random DNA probes labelled with [α-^{32}P]dCTP and diluted transcripts constituted positive controls (+C) (a, b). The 19 kDa CP and 115 kDa CP–RT proteins of BSBV were immuno-detected with primary polyclonal antibody directed against viral particles (c, d).

All three replicative combinations raised the infection rate by 100% in both host plants, except the 1+2*+3 which infected half of the inoculated *B. macrocarpa*.

BSBV CP and CP–RT proteins were detected by Western blotting after SDS-PAGE separation of total protein extracts from symptomatic samples and whole asymptomatic leaves. Structural proteins were revealed using a polyclonal antibody directed against viral particles (DSMZ). The primary antibody was diluted 1 : 10 000 in TBS buffer containing 0.1% Tween 20, 5% powdered skimmed milk, and supplemented with total protein extracts from healthy *C. quinoa* or *B. macrocarpa*. Anti-rabbit alkaline phosphatase-conjugated secondary IgG (Sigma-Aldrich) was diluted 1 : 10 000 in TBS–Tween–milk buffer and the blots were revealed with premixed BCIP–NBT solution (Sigma-Aldrich).

A 19 kDa protein was detected in both *C. quinoa* and *B. macrocarpa* plants inoculated with the 1+2*+3 and 1+2+3 mixes of BSBV RNAs transcripts (Fig. 3c, d, lanes 1 and 2) but not when RNA-2 was omitted (combination 1+3; Fig. 3c, d, lane 4). Thus, the BSBV RNA-2 transcripts are indeed responsible for the expression of the 19 kDa protein *in planta*, which corresponds to the BSBV CP of the same predicted molecular mass that is encoded by the BSBV RNA-2 first ORF. Another protein with an estimated molecular mass of 115 kDa was also observed in the leaves of both plants inoculated with the genome combination 1+2+3 (Fig. 3c, d, lane 2) and not detected in the leaves inoculated with the BSBV RNA-2 transcripts mutated for the RT domain of the CP (genome combination 1+2*+3; Fig. 3c, d, lane 1). This product therefore corresponds to the predicted 104 kDa BSBV CP–RT protein. Transcript progeny analysis confirmed the stability of the frame shift mutation (data not shown).

According to the different genomic combinations of BSBV full-length transcripts inoculated, our study showed that both the putative replicase (RNA-1) and TGB proteins (RNA-3) of BSBV are essential and sufficient for the replication of such RNAs in the inoculated leaves of *C. quinoa* and *B. macrocarpa*. In addition, the symptom expression on *C. quinoa* did not depend on the presence of the BSBV RNA-2. The BSBV CP and CP–RT encoded by the BSBV RNA-2 are therefore not required for viral infection in the plant hosts or for symptom production on *C. quinoa* leaves. The dispensability of the CP for viral replication *in planta* is a common phenomenon, shared by the closely related pomovirus potato mop-top virus (PMTV) on *Nicotiana benthamiana* and *Nicotiana clevelandii* (McGeachy & Barker, 2000; Savenkov *et al.*, 2003), as well as other viruses such as the well-known tobamovirus tobacco mosaic virus (TMV) on *Nicotiana tabacum* (Siegel *et al.*, 1962). However, whereas symptom production due to TMV does not depend on the presence of the CP, no symptoms are expressed when PMTV lacks the CP and CP–RT. Thus, despite their taxonomic proximity and a highly similar genome organization, the BSBV and PMTV pomoviruses retain their own particularities. Nevertheless, it should be borne in mind that PMTV is a potato virus and symptom expression could be host-dependent.

It is also worth noting that, as the primers used to amplify full-length cDNA sequences of BSBV were designed according to a previously published nucleotide sequence of a German isolate, the biological properties of the resulting full-length cDNA clone might have been modified. However, viral terminal sequences are mostly highly conserved for a virus species as they are involved in viral replication (Duggal *et al.*, 1994; Koenig *et al.*, 2000), and the BSBV full-length clone replicated efficiently.

Consistent with earlier findings reporting on foliar symptoms on *Chenopodium* spp. and occasional lesions on *Beta* spp. leaves when inoculating BSBV from the crude sap of infected plants (Henry *et al.*, 1986), transcripts from the full-length clone of BSBV induced clear symptoms only on the *C. quinoa* leaves. Following the example of BSBV, the two first BNYVV RNAs, carrying the genetic material homologous to one of the three BSBV RNAs plus a cysteine-rich protein (p14) with PTGS suppressor activity (Bouzoubaa *et al.*, 1986, 1987; Dunoyer *et al.*, 2002), are also unable to induce a systematic symptomatic foliar reaction on *Beta* spp. However, lesions on *Beta* spp. leaves are clearly produced when at least BNYVV RNAs-1, -2 and -3 are present (Tamada *et al.*, 1989; Rahim *et al.*, 2007). Although BSBV and BNYVV both naturally infect sugar beet, BSBV lacks the genetic material necessary to induce clear symptoms on *B. macrocarpa* leaves whereas BNYVV has this through RNAs-3 and -4.

The use of a polyclonal antibody directed against BSBV viral particles allowed two proteins expressed from BSBV RNA-2 transcripts to be detected. As expected, the Western blot analysis showed the production of the BSBV 19 kDa CP in both *C. quinoa* and *B. macrocarpa*. However, whereas the expected molecular mass of the CP–RT is 104 kDa, a protein with an estimated molecular mass of 115 kDa corresponding to the CP–RT was identified in both host plants. It could be assumed that post-translational modifications of the CP–RT domain, such as phosphorylation or glycosylation events, are responsible for this 115 kDa observed molecular mass of CP–RT. Actually, post-translational modifications can reduce the protein's electrophoretic mobility in SDS-PAGE and many viral structural proteins are described as glycosylated and/or phosphorylated (Hahn & Shepherd, 1980; Seddas & Boissinot, 2006; Akamatsu *et al.*, 2007; Hafrén & Mäkinen, 2008; Zayakina *et al.*, 2008). Another hypothesis would involve the intrinsic properties of the BSBV CP–RT, knowing that highly hydrophilic domains containing many charged residues, such as arginine (Hu & Ghabrial, 1995), or the presence of transmembrane motifs (Rath *et al.*, 2009) in proteins might influence their migration on SDS-PAGE. This is supported by the presence in the RT domain of the BSBV CP of two predicted transmembrane helices, as is also reported for the closely related beet virus Q (BVQ)

(Crutzen *et al.*, 2009), which would be essential for viral transmission by the vector *P. betae* (Diao *et al.*, 1999; Adams *et al.*, 2001). Further experiments need to be performed to determine the specificity of CP–RT, from the post-translational modification or intrinsic properties, which influences the molecular mass of the protein on SDS-PAGE.

The rhizomania syndrome of sugar beet and its causal agent, BNYVV, have been widely studied since the 1970s because cropping losses, commonly about 50 % and sometimes up to 80 %, have been attributed to the disease (McGrann *et al.*, 2009). However, the involvement of BVQ and BSBV in the disease, often associated with beet infected with BNYVV, has never been clearly established and no particular interest in understanding their specific functionality was followed. Our study describing the first infectious full-length cDNA clone available for a beet *Pomovirus* therefore provides a useful tool for further investigating the pathogenicity of BSBV in the complex rhizomania syndrome, as well as its replication and infection mechanisms, and the potential viral interactions with other beny- and pomoviruses on both susceptible and rhizomania-resistant beets.

Acknowledgements

F.C. is a research fellow at the Belgian National Fund of Scientific Research (FNRS) and M.M. is supported by grants from the Ministry of Science, Research and Technology, Iran.

References

Adams, M. J., Antoniw, J. F. & Mullins, J. G. L. (2001). Plant virus transmission by plasmodiophorid fungi is associated with distinctive transmembrane regions of virus-encoded proteins. *Arch Virol* **146**, 1139–1153.

Akamatsu, N., Takeda, A., Kishimoto, M., Kaido, M., Okuno, T. & Mise, K. (2007). Phosphorylation and interaction of the movement and coat proteins of *Brome mosaic virus* in infected barley protoplasts. *Arch Virol* **152**, 2087–2093.

Bouzoubaa, S., Ziegler, V., Beck, D., Guilley, H., Richards, K. & Jonard, G. (1986). Nucleotide sequence of beet necrotic yellow vein virus RNA-2. *J Gen Virol* **67**, 1689–1700.

Bouzoubaa, S., Quillet, L., Guilley, H., Jonard, G. & Richards, K. (1987). Nucleotide sequence of beet necrotic yellow vein virus RNA-1. *J Gen Virol* **68**, 615–626.

Canova, A. (1959). On the pathology of sugar beet. *Inf Fitopatol* **9**, 390–396.

Crutzen, F., Kreit, M. & Bragard, C. (2009). The *Beet virus Q* coat protein readthrough domain is longer than previously reported, with two transmembrane domains. *J Gen Virol* **90**, 754–758.

Diao, A., Chen, J., Gitton, F., Antoniw, J. F., Mullins, J., Hall, A. M. & Adams, M. J. (1999). Sequences of *European wheat mosaic virus* and *Oat golden stripe virus* and genome analysis of the genus *Furovirus*. *Virology* **261**, 331–339.

Duggal, R., Lahser, F. C. & Hall, T. C. (1994). *cis*-Acting sequences in the replication of plant viruses with plus-sense RNA genomes. *Annu Rev Phytopathol* **32**, 287–309.

Dunoyer, P., Pfeffer, S., Fritsch, C., Hemmer, O., Voinnet, O. & Richards, K. E. (2002). Identification, subcellular localization and some properties of a cysteine-rich suppressor of gene silencing encoded by *Peanut clump virus*. *Plant J* **29**, 555–567.

Hafrèn, A. & Mäkinen, K. (2008). Purification of viral genome-linked protein VPg from *Potato virus A*-infected plants reveals several post-translationally modified forms of the protein. *J Gen Virol* **89**, 1509–1518.

Hahn, P. & Shepherd, R. J. (1980). Phosphorylated proteins in *Cauliflower mosaic virus*. *Virology* **107**, 295–297.

Henry, C. M., Jones, R. A. C. & Coutts, R. H. A. (1986). Occurrence of a soil-borne virus of sugar beet in England. *Plant Pathol* **35**, 585–591.

Hu, C.-C. & Ghabrial, S. A. (1995). The conserved, hydrophilic and arginine-rich N-terminal domain of cucumovirus coat proteins contributes to their anomalous electrophoretic mobilities in sodium dodecylsulfate-polyacrylamide gels. *J Virol Methods* **55**, 367–379.

Hull, R. (2009). How do plant virus work? Virus–plant interactions: molecular level. In *Comparative Plant Virology*, 2nd edn, pp. 191–206. London: Academic Press.

Ivanović, M., Macfarlane, I. & Woods, R. D. (1983). Viruses of sugar beet associated with *Polymyxa betae*. In *Annual Report of Rothamsted Experimental Station for 1982*, pp. 189–190. Harpenden, UK: IARC Rothamsted.

Jupin, I., Richards, K., Jonard, G., Guilley, H. & Pleij, C. W. (1990). Mapping sequences required for productive replication of *Beet necrotic yellow vein virus* RNA 3. *Virology* **178**, 273–280.

Kaufmann, A., Koenig, R. & Rohloff, H. (1993). Influence of beet soil-borne virus on mechanically inoculated sugar beet. *Plant Pathol* **42**, 413–417.

Koenig, R. & Loss, S. (1997). Beet soil-borne virus RNA 1: genetic analysis enabled by a starting sequence generated with primers to highly conserved helicase-encoding domains. *J Gen Virol* **78**, 3161–3165.

Koenig, R., Beier, C., Commandeur, U., Lüth, U., Kaufmann, A. & Lüddecke, P. (1996). Beet soil-borne virus RNA 3 – a further example of the heterogeneity of the gene content of furovirus genomes and of triple gene block-carrying RNAs. *Virology* **216**, 202–207.

Koenig, R., Commandeur, U., Loss, S., Beier, C., Kaufmann, A. & Lesemann, D.-E. (1997). Beet soil-borne virus RNA 2: similarities and dissimilarities to the coat protein gene-carrying RNAs of other furoviruses. *J Gen Virol* **78**, 469–477.

Koenig, R., Pleij, C. W. A. & Büttner, G. (2000). Structure and variability of the 3′ end of RNA 3 of *Beet soil-borne pomovirus* – a virus with uncertain pathogenic effects. *Arch Virol* **145**, 1173–1181.

Lauber, E., Guilley, H., Tamada, T., Richards, K. E. & Jonard, G. (1998). Vascular movement of beet necrotic yellow vein virus in *Beta macrocarpa* is probably dependent on an RNA 3 sequence domain rather than a gene product. *J Gen Virol* **79**, 385–393.

Lindsten, K. (1993). Rhizomania – are both *Beet necrotic yellow vein virus* (BNYVV) and *Beet soil-borne virus* (BSBV) involved? In *Proceedings of the Second Symposium of the International Working Group on Plant Viruses with Fungal Vectors*, 2nd edn, pp. 67–70. Edited by C. Hiruki, American Society of Sugar Beet Technologists, Denver.

McGeachy, K. D. & Barker, H. (2000). *Potato mop-top virus* RNA can move long distance in the absence of coat protein: evidence from resistant, transgenic plants. *Mol Plant Microbe Interact* **13**, 125–128.

McGrann, G. R., Grimmer, M. K., Mutasa-Göttgens, E. S. & Stevens, M. (2009). Progress towards the understanding and control of sugar beet rhizomania disease. *Mol Plant Pathol* **10**, 129–141.

Meunier, A., Schmit, J.-F., Stas, A., Kutluk, N. & Bragard, C. (2003). Multiplex reverse transcription-PCR for simultaneous detection of *Beet necrotic yellow vein virus*, *Beet soilborne virus* and *Beet virus Q* and their vector *Polymyxa betae* KESKIN on sugar beet. *Appl Environ Microbiol* **69**, 2356–2360.

Prillwitz, H. & Schlösser, E. (1992). Beet soil-borne virus: occurrence, symptoms and effect on plant development. In 44th International Symposium on Crop Protection. Ghent, Belgium, May 1992, pp. 295–302.

Rahim, M. D., Andika, I. B., Han, C., Kondo, H. & Tamada, T. (2007). RNA4-encoded p31 of beet necrotic yellow vein virus is involved in efficient vector transmission, symptom severity and silencing suppression in roots. *J Gen Virol* **88**, 1611–1619.

Rath, A., Glibowicka, M., Nadeau, V. G., Chen, G. & Deber, C. M. (2009). Detergent binding explains anomalous SDS-PAGE migration of membrane proteins. *Proc Natl Acad Sci U S A* **106**, 1760–1765.

Rush, C. M. & Heidel, G. B. (1995). Furovirus diseases of sugar beets in the United States. *Plant Dis* **79**, 868–875.

Savenkov, E. I., Germundsson, A., Zamyatnin, A. A., Jr, Sandgren, M. & Valkonen, J. P. (2003). *Potato mop-top virus*: the coat protein-encoding RNA and the gene for cysteine-rich protein are dispensable for systemic virus movement in *Nicotiana benthamiana*. *J Gen Virol* **84**, 1001–1005.

Seddas, P. & Boissinot, S. (2006). Glycosylation of *Beet western yellows virus* proteins is implicated in the aphid transmission of the virus. *Arch Virol* **151**, 967–984.

Siegel, A., Zaitlin, M. & Sehgal, O. P. (1962). The isolation of defective *Tobacco mosaic virus* strains. *Proc Natl Acad Sci USA* **48**, 1845–1851.

Tamada, T. & Baba, T. (1973). *Beet necrotic yellow vein virus* from rhizomania-affected sugar beet in Japan. *Ann Phytopathol Soc Jpn* **39**, 325–332.

Tamada, T., Shirako, Y., Abe, H., Saito, M., Kiguchi, T. & Harada, T. (1989). Production and pathogenicity of isolates of Beet necrotic yellow vein virus with different numbers of RNA components. *J Gen Virol* **70**, 3399–3409.

Zayakina, O., Arkhipenko, M., Kozlovsky, S., Nikitin, N., Smirnov, A., Susi, P., Rodionova, N., Karpova, O. & Atabekov, J. (2008). Mutagenic analysis of potato virus X movement protein (TGBp1) and the coat protein (CP): *in vitro* TGBp1-CP binding and viral RNA translation activation. *Mol Plant Pathol* **9**, 37–44.

X.2. Distribution and characterization of Iranian *Beet black scorch Virus*

(Proceeding of 7th international working group on plant viruses with fungal vectors)

DISTRIBUTION AND CHARACTERIZATION OF IRANIAN *BEET BLACK SCORCH VIRUS*

Mohsen Mehrvar[1,2] and Claude Bragard[2]

[1] Ferdowsi University of Mashhad, Department of Plant Protection, School of Agriculture, Mashhad, Iran
[2] Université catholique de Louvain, Unite de Phytopathologie, B-1348 Louvain-la-Neuve, Belgium

Summary

The Iranian provinces where sugar beet is cultivated have been surveyed for the presence of *Beet black scorch virus*. Sixty two samples (31%) out of two hundreds and three samples showed positive response by RT-PCR in ten out of thirteen Iranian provinces of Khorasan Shomali, Khorasan Razavi, Ghazvine, Zanjan, Ardabil, Azarbijaan Sharghi, Azarbijaan Gharbi, Kermanshah, Hamadan and Kerman. The BBSV satellite was found in nineteen samples (9.3 %) out of 203 in all provinces except Azarbaeijan Shargi. The presence of BBSV was systematically associated by the presence of BNYVV and/or other soil-borne pomoviruses. The complete genome sequences of three Iranian BBSV isolates have been determined. The three isolates, one with satellite designated BBSV-Ha1 and two without satellite designated BBSV-AzGh and BBSV-Ha2 showed 97 % nucleotide identity between the first two and 88 % between the two first and third one. The identity between two Iranian isolates of BBSV-AzGh, BBSV-Ha1 and one isolate of BBSV-Ha2 by both genomes of the 'Ningxia' isolate of BBSV (BBSV-N) from China and 'BBSV-Co' from US were 89 % and 88 % respectively in nt level of whole genomes. In all of the cases Iranian BBSV was accompanied by BNYVV causal agent of rhizomania.

Introduction

Beet black scorch virus (BBSV) was first identified in Inner Mongolia, China, in the late 1980s (Cui *et al*., 1988; Liu and Xian, 1995; Zhang *et al*., 1996). It was described as a new *Necrovirus* species (Cao *et al*., 2002; Lommel *et al*., 2005). Recently it was reported from US (Weiland *et al*., 2007) and from Iran (Koenig and Valizadeh, 2008).
The virus elicits severe, systemic disease symptoms described as black scorched leaves of sugar beet (*Beta vulgaris* L.) in China. The BBSV isolates reported from US did not show such a black scorching in leaves, but exacerbate symptoms of Rhizomania like rootlet proliferation (Weiland *et al*., 2007). Symptomless infections have been observed on sugar beet. The virus can be detected in each of these hosts by RT-PCR and by retro-inoculation to *Chenopodium quinoa*.
BBSV is belonging to the *Tombusviridae* family. The virus is transmitted efficiently through the soil in a non-persistent manner by zoospores of *Olpidium brassicae* (Jiang *et al*., 1999). The virus comprises an icosahedral particle of 28 nm that encapsidates a positive-sense, single-stranded (ss) genomic (g) RNA of circa 3644 nt. BBSV shares the highest sequence identity (61%) with *Tobacco necrosis virus* D (TNV-D) (Coutts *et al*., 1991).
Normally, only a single RNA is associated with infections, but a small single-stranded RNA (ssRNA) that is similar to the satellite RNAs (sat-RNA) of other necroviruses has been found in isolates from the Xinjiang province of China (Guo *et al*., 2005).

Up to date, the extend of BBSV distribution has not been reported. The aim of the present study is to appraise the BBSV and its satellite distribution in Iran, with a special attention to other *Polymyxa*-transmitted soil-borne viruses of sugar beet.

Materials and methods

Plant material and virus isolates

203 sugar beet root samples were collected either directly from the fields or from bioassays (Meunier *et al*., 2003). Samples were localized by GPS (Global Positioning System)

and a picture from the field sampled as well as from the gathered samples was taken. Rootlets from infected sugar beet and single chlorotic lesion of *C. quinoa* leaves were used for RNA extraction using a SV Total RNA Extraction Kit (Promega). Approximately 171 mg of rootlets or leaves were ground in 1000 µL of lysis buffer in a sterile pestle and mortar (According to Kit Instruction adjustments) and RNA extracted as described in the manufacturer's manual: RNAs were used directly or stored at minus 70 °C.

RT-PCR and Sequencing

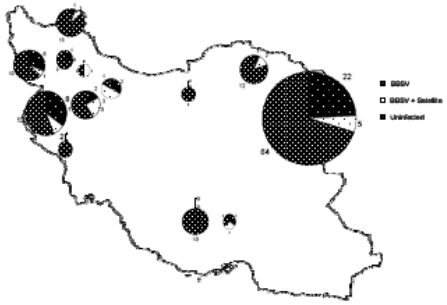
For the detection of BBSV, primers by Weiland *et al.* (2007) were used, which amplify a fragment of 315 bp. within the 3' UTR of BBSV RNA. Two hundred nanograms of purified PCR product (DNA), 1 µL of primer (4 µM) and 4 µL of Biomol. Water (Amersham Pharmacia Biotech) were mixed and sequenced. Sequences were analyzed by the Clustal W program of the Genetic Computer Group.

Viral genome characterization and sequence comparison

Plasmid pBBSV-Ha1, pBBSV-Ha2, pBBSV-Msh, pBBSV-AzGh, pBBSV-Ksh were subjected to double-stranded sequencing using primers positioned at an average of 400-bp intervals and the sequence assembled using vector NTI Advance 10 program (Invitrogen) and ORF finder program (NCBI), Major ORFs present within the sequence of BBSV-Ha1, BBSV-Ha2, pBBSV-Msh, BBSV-AzGh, pBBSV-Ksh were detected using NTI Advance 10 program (Invitrogen) and ORF finder program (NCBI). Sequence alignments were performed using Clustal W.

Results

In September 2006, a survey was carried out in all sugar beet growing areas in Iran, to assess the current of rhizomania. When the 203 gathered samples were tested for the presence of BBSV, the virus was found in 62 (31 %) of the tested Iranian sugar beet field. It was distributed in Khorasan Shomali, Khorasan Razavi, Zanjan, Ghazvine, Ardabil, Azarbaeijan Sharghi, Azarbaeijan Gharbi, Kermanshah, Hamadan and Kerman (Fig. 1). In terms of symptoms mostly it was mixed by BNYVV symptoms which are root proliferation but in greenhouse some extend of leaves black scorch on sugar beet leaves just in BBSV-AzGh isolate appeared.



Also the satellite of BBSV was found in the nine provinces of Ardabil, Azarbaeijan Gharbi, Ghazvine, Zanjan, Hamadan, Kerman, Kermanshah, Khorasan Razavi and Khorasan shomali. In some cases we have seen some huge damage to the sugar beet production in the field because of a strong presence of BNYVV, BSBV, BVQ and BBSV which may explain a synergistic affect on sugar beet yet to be established.

Fig. 1 : Distribution and number of infected and uninfected sugar beet samples by BBSV, BBSV satellites in different Iranian provinces, proportionally to the extend of the sugar beet cultivated area

The highest incidence rate were found in the Khorasan Razavi (35%) and Kermanshah (16 %) provinces followed by Khorasan Shomali (11 %), Azarbaeijan Gharbi (9.6 %), Hamadan (8 %) and Ghazvine (8 %). Ardabil and Kerman (3.2 %) and Zanjan and Azarbaeijan Sharghi (1.6 %) were less affected by the virus (Fig. 1).

Mechanical inoculation of *C. quinoa* resulted in the production of the typical necrotic local lesions with chlorotic halos at three to five days post inoculations, but occasionally extended in necrotic areas on most of the inoculated leaves, also in BBSV-AzGh isolate symptoms were more systemic and extended along with leaves main veins. Transfer of the infection to leave of sugar beet resulted in no symptoms. The complete nucleotide sequences and genome organization of BBSV-AzGh, BBSV-Ha2 and BBSV-Ha1 as well as it's satellite were determined and compared with that of BBSV-CO, BBSV-N, -X, and other viruses. Linear alignments of the 3,644-nt genomes of BBSV-AzGh, BBSV-Ha1 with BBSV-CO, BBSV-N and BBSV-X using clustal W indicated a 89 % identity between the genome of these virus isolates, For BBSV-Ha2 it was 88%.

We detected also the satellite of BBSV in Iranian BBSV-Ha1. This BBSV satellite has already reported from China but not from US isolate. The satellite was detected in 30% of the samples positive for the presence of BBSV. Iranian BBSV was mostly accompanied by BNYVV, the casual agent of sugar beet Rhizomania.

Discussion

Up to now, BBSV has only been found in China, Iran and U.S.A. (Weiland, 2007; Koenig, 2008). To date, there is no data publicly available on the distribution of the virus and its economical importance, though, by comparison with BNYVV, another soil-borne virus of sugar beet, it might be inferred as of strategic stake for the future, especially in warmer areas. The recent discovery of the BBSV raised also the question of its origin. In this study, the widespread occurrence of BBSV, alone or with its satellite, in most sugar beet growing areas of Iran was assessed by bioassays and RT-PCR. The virus is present in 10 provinces and was recorded in 31 % of the samples analyzed while the satellite was detected in only 8.8%.
The analysis of the BBSV diversity in Iran reveals a wider diversity than the one reported from China or the U.S.A.

Approximately ¼ of the BBSV-containing samples found in Iran are associated with the virus satellite. Such satellite has been found to enhance the symptom expression, though the symptoms observed were mostly similar to rhizomania symptoms, instead of the beet black scorch symptoms initially described by Cao *et al.* (2002). Weiland *et al.* (2007) did observe similar results in the States, though the sugar beet in their trials were not contaminated by BNYVV. This raised the question of the presence of other soil-borne viruses in co-infection with the BBSV and its satellite.

In Iran, BBSV was always found together with other *Polymyxa*-transmitted soil-borne viruses. Mostly, BBSV is associated with *Beet necrotic yellow vein virus* alone or in combination with sugar beet infecting pomoviruses, *Beet soil-borne virus* and *Beet virus Q*. Such association is possibly an explanation for the observation of the root beardedness also noted by Koenig and Valizadeh (2008) and Weiland *et al.* (2007). The co-occurrence of up to four different soil-borne viruses is also raising questions about potential synergism or competition.

In terms of distribution, the prevalence of BNYVV is much stronger than the one of BBSV. A possible explanation for this is the difference of vector between both viruses. Though the lack of information on the host range and potential biotypes of *Olpidium* found in association with sugar beet, several reports mention the association. Interestingly, several reports have also documented TNV occurrence on sugar beet in the past, and it might be worth exploring if these were not related with BBSV, since a weak cross reaction between both viruses was found by ISEM (Cao *et al.*, 2002).

The quasi absence of BBSV infection in the warmer provinces of Fars is possibly due to the fact that *O. brassicae* zoospore are less tolerant to warmer temperature than other *Olpidium* (Gharbi and Verhoyen, 1993, Campbell and Lin, 1976).

References

Bo, Y.-X., Cai, Z.-N., Ding, Q., Yu, J.-L. and Liu, Y. (1996). *Characterization of beet black scorch virus* RNA, partial cDNA cloning and sequencing of the small component. J Agric Biotechnol **4**, 269–276.

Cai, Z.-N., Chen, D.-H., Wu, M.-S., Cui, X.-M., Yu, J.-L. and Liu, Y. (1993). Identification of pathogenic virus of beet black scorch disease and detection by synthesized cDNA probes. J Beijing Agric Univ **19**, 112.

Campbell, R. N., and Lin, M. T. (1976). Morphology and thermal death point of *Olpidium brassicae*, American Journal of Botany **63**, 826–832.

Cao, Y.-H., Cai, Z.-N., Ding, Q., Li, D.-W., Han, C.-G., Yu, J.-L. and Liu, Y. (2002). The complete nucleotide sequence of *Beet black scorch virus* (BBSV), a new member of the genus Necrovirus. Arch Virol **147**, 2431–2435.

Coutts, R. H., Rigden, J. E., Slabas, A. R., Lomonossoff, G. P., and Wise, P. J. (1991). The complete nucleotide sequence of tobacco necrosis virus strain D. J Gen Virol **72**, 1521–1529.

Cui, X.-M., Gong, Z.-X. and Yu, N.-J. (1988). Studies on two virus isolates from sugar beet in Xinjiang. J Shihezi Agric Coll 10, 73–78.

Gharbi, S. and Verhoyen, M. (1993). Sterilization by UV irradiation of nutrient solutions with a view to avoiding viral infections transmitted by *Olpidium brassicae* in hydroponic culture of lettuce. Mededelingen Faculteit Landbouwwetenschappen. *Universiteit Gent* **58**, 1113–1124.

Guo, L.-H., Cao, Y.-H., Li, D.-W., Niu, S.-N., Cai, Z.-N., Han, C.-G., Zhai, Y.-F. and Yu, J.-L. (2005). Analysis of nucleotide sequences and multimeric forms of a novel satellite RNA associated with *beet black scorch virus*. J Virol **79**, 3664–3674.

Jiang, J.-X., Zhang, J.-F., Che, S.-C., Yang, D.-J., Yu, J.-L., Cai, Z.-N. and Liu, Y. (1999). Transmission of *beet black scorch virus* by Olpidium brassicae. J Jiangxi Agric Univ **21**, 525–528.

Koenig, R., Valizadeh, J. (2008). Molecular and serological characterization of an Iranian isolate of Beet black scorch virus. Arch Virol 153, 1397-1400.

Liu, J.-X. & Xian, H.-Q. (1995). Preliminary report on Beet black scorch virus. China Sugarbeet **3**, 30–31.

Lommel, S. A., Martelli, G. P., Rubino, L. and Russo, M. (2005). Tombusviridae. In Virus Taxonomy. Eighth Report of the International Committee on Taxonomy of Viruses, pp. 906–936. Edited by C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger & L. A. Ball. London: Elsevier/Academic Press.

Meunier, A., Schmit, J. F., Stas, A., Kutluk, N. and Bragard, C. (2003). Multiplex RT-PCR for the simultaneous detection of *Beet necrotic yellow vein virus*, *Beet soilborne virus*, *Beet virus Q* and their vector *Polymyxa betae* KESKIN on sugar beet. Appl Environ Microbiol **69**, 2356-2360.

Weiland, J., Winkle, D., Edwards, M. C., Larson, R. L., Shelver, W. L., Freeman, T. P., and Liu, H. (2007). Characterization of a U.S. Isolate of *Beet black scorch virus*. October Phytopathol.ogy **97**, 1245-1254.

Zhang, K.-W., Wang, S.-L., Cai, Z.-N. and Zhang, H.-S. (1996). Preliminary study on beet black scorch disease in Ningxia Province. China Beet Sugar **2**, 3–8.

X.3. Material and methods

X.3.1. *Beet black scorch virus* (BBSV) molecular analysis

X.3.1.1 RT-PCR

For the detection of BBSV, primers by Weiland *et al.* (2007) were used, which amplify a fragment of 315 bp. within the 3' UTR of BBSV RNA. Primer sequences and respective nucleotide positions in BBSV RNA are shown in Table 4 (Weiland *et al.*, 2007). The reverse transcription (RT) reaction was performed as follows: 1 µl of reverse primer (20 picomoles) and 1 µl of RNA sample were added to 8.5 µl of DEPC-treated water. The mixture was incubated at 65 °C for 10 minutes and directly transferred onto ice. Then, 3.5 µl of DEPC-treated water, 4 µl of 5X M-MLV RT buffer, 2 µl of dNTPs mix (10 mm) and 0.25 µl of M-MLV (200 U µl) reverse transcriptase (Promega) were added to the mixture. The RT reaction was performed at 42 °C for 60 minutes. The PCR reaction involved: 2.5 µl of cDNA, 13.25 µl of DEPC-treated water, 5µl of 5X *GoTaq* polymerase buffer (Promega), 2.5 µl of 10X $MgCl_2$ (Promega), 0.5 µl each of forward and reverse primers (20 picomoles µ L), 0·75 µl of dNTPs mix (10 mm) and 0.125 µl of *GoTaq* polymerase (2.5 U µl) (Promega). A first denaturation of 3 minutes at 94 °C was performed, then 35 cycles of: denaturation for 30 seconds at 94 °C, annealing for 30seconds at corresponding temperature for each set of primers which is mention in Table 4 and elongation for 2 minutes at 72 °C, followed by a final elongation step of 7 minutes at 72 °C. To produce a PCR product for sequence analysis, primers BBSV1F and BBSV1R were used. After the PCR, 25 µl of the reaction mix was loaded onto a 1.5% agarose gel, subjected to electrophoresis, and cDNA was stained with ethidium bromide. PCR products were purified using a High Pure PCR Product Purification Kit (Qiagen) and then sequenced. Then different set of primers for sequencing of full-length genome of BBSV as well as its satellite were designed according to BBSV-N sequence in database (Yuan *et al.*, 2006) and by use of eprimer3 program in Belgian EMBnet Node.

X.3.1.2 Determination of 3’ ends

The 3’end of viral RNA was polyadenylated with Poly (A) polymerase (Takara biotech, Shiga, Japan). The polyadenylated RNA was used as template for cDNA synthesis by using Oligo(dT)16

anchor as the primer. PCR was conducted with Oligo(dT)16 anchor and sequence specific primer BBSV8. The PCR product inserted in pGEM-T Vector and sequenced (Dehui *et al.*, 2008) (Fig. 14).

X.3.1.3 Sequence analysis

For the sequence analysis, the forward and reverse primers in table 4 were used as follows: Two hundred nanograms of purified PCR product (DNA), 0.25 µL of primer (20 µM) and 10.5 µL biomol water (Amersham Pharmacia Biotech) were mixed and sequenced using an ABI377 sequencer (Applied Biosystems). Sequences were analyzed by the Clustal W program of the Genetic Computer Group (Devereux *et al.*, 1984)

X.3.1.4 BBSV RNA characterization and full-length cloning

The Expand Reverse Transcriptase kit (Roche), which includes a high-fidelity, thermo- stable polymerase, was used to generate a complete cDNA fragments from the virion RNA.
Primer designated BBT75fwd and BBSV3rev (Weiland *et al.*, 2007) were used for the amplification of complete BBSV genome-length amplicon using the Expand Long Template PCR System kit (Roche). RNA extractions of seven different Iranian BBSV isolates were obtained. The RT-PCR was done by a mixed of 4.5 µl of diethyl pyrocarbonate (DEPC)-treated water, 1,2 µl of BBSV1 reverse primer and 4 µl of virus RNA in 65 °C for 10 minutes. Then 9.5 µl of mixed solution (0.5 µl of diethyl pyrocarbonate (DEPC)-treated water, 4 µl Buffer, 2 µl of DTT, 2 µl of DNTPs and 1 µl RT (Reverse Transcriptase) Enzyme) was added and put in 43°C for 1 hour.
The long expand PCR was done by adding 2,5 µl of RT product to 47,5 µl of a mixed solution (38,9 µl diethyl pyrocarbonate (DEPC)-treated water, 4 µl Buffer1, 1,75 µl DNTPs, 0,8 µl of each forward and reverse primers and 0,75 µl of long expand polymerase) and put in thermocycler in following conditions: 94°C for 2 minutes (1 cycle), 10 cycles in 94°C for 10 seconds, 52,9°C for 10 seconds, 68°C for 2 minutes, 28 cycles in 94°C for 15 seconds, 52,9°C for 30 seconds, 68°C for 2min+20secods cycle elongation for each successive cycle and finally 1 cycle in 68°C for 7 minutes. The corresponding bands for full-length BBSV revealed (3644 bp.) after the long expand PCR product run on agarose gel (1%) and staining by ethidium bromide (Fig. 7).

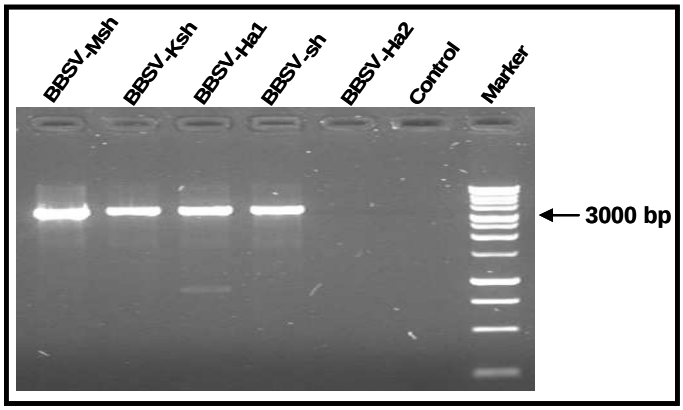


Fig. 7 Full-length amplicon of four Iranian BBSV-Ha1, BBSV-Ksh1, BBSV-Msh1 and BBSV-Sh1 isolates.

X.3.1.5 Cloning of full-length product of BBSV in pDrive and pGMT vectors

Different Iranian BBSV full-length amplicons were cloned in both pDrive and pGMET vectors (Fig. 14, 15). Inserted fragments selected and amplified in liquid LB (containing ampicilin) medium for 16 hours. DNA was extracted from plasmid vector by Mini prep kit (Qiagen).

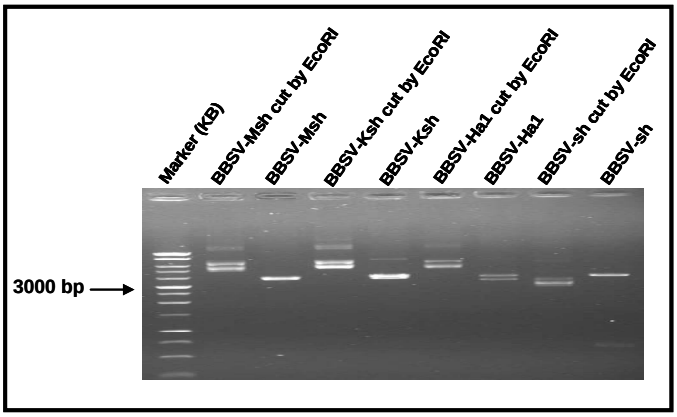


Fig. 8 Restriction of full-length fragment of four Iranian BBSV-Ha1, BBSV-Ksh1, BBSV-Msh1 and BBSV-Sh1 isolates by EcoRI restriction Enzymes

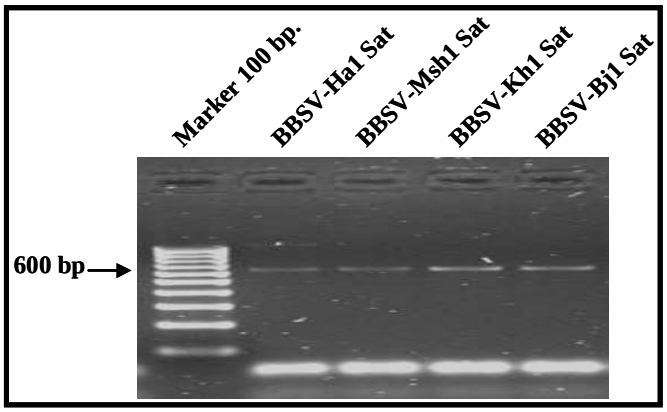


Fig. 9 Full-length amplification of different Iranian BBSV satellites (BBSV-Ha1sat, BBSV-Msh1sat, BBSV-Kh1sat and BBSV-Bj1sat)

X.3.1.6 Viral genome characterization and sequence comparison.

Plasmid pBBSV-AzGh1, pBBSV-Ha1, pBBSV-Ha2, pBBSV-Ksh1, pBBSV-Msh1, pBBSV-Sh1 and BBSV-Ksh3 were subjected to double-stranded sequencing using primers positioned at an average of 400-bp intervals and the sequences assembled using vector NTI Advance 10 program (Invitrogen) and ORF finder program (NCBI), Sequence alignments and inference of genetic distance were performed using Clustal W (Thompson *et al*., 1994).

X.3.1.7 *Beet black scorch virus* RNA transcription in vitro

Different Iranian BBSV isolates cloned in pDrive vector (Qiagene, Germany), digested by EcoR1 restriction enzyme leading to liberation of the inserted clone from the plasmid vector (Fig. 8), providing a template from which transcription would initiate one G nucleotide before the viral sequence. Transcription reaction with T7 RNA polymerase for generating uncapped RNA was used to produce genome-length RNA for inoculation to plant leaves (Fig. 10).

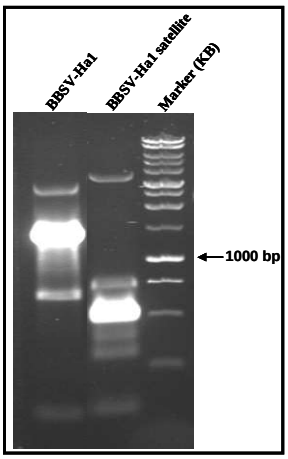


Fig. 10 Transcription of Iranian BBSV-Ha1 and BBSV-Ha1sat constructs by RiboMAX T7 kit.

X.3.1.8 Inoculation of BBSV transcript in host plants

Infectivity of the transcript RNA was tested by suspending 2 to 5 µg of RNA in GKP buffer (50 Mm glycine, 30 mM K2HPO4, 1% bentonite, and celite, pH 9.2) and mechanically inoculated on leaves of *C. quinoa* and sugar beet (Julietta, Sofia and Cadyx) (Weiland & Edwards, 1994).

X.3.2. Iranian *Beet soil-borne virus* molecular analysis

X.3.2.1 Viral RNA characterization and full-length cloning of Iranian *Beet soil-borne virus*

The Expand Reverse Transcriptase kit (Roche), which includes a high-fidelity, thermo-stable polymerase, was used to generate a complete cDNA fragments from the virion RNA. as follows: Primer designated BSBV1, BSBV2 and BSBV3 were used for the amplification of complete BSBV genome-length amplicon of RNA1, RNA2 and RNA3 of one Iranian BSBV-Ny isolate. The RT-PCR was done by 8.5 µl of diethyl pyrocarbonate (DEPC)-treated water, 1 µl of reverse primer and 1 µl of RNA put it in thermocycler in 65 °C for 10 min. then 9.5 µl of mixed buffer (0.5 µl of diethyl pyrocarbonate (DEPC)-treated water, 4 µl of RT Buffer, 2 µl of DTT, 2 µlof DNTPs and 1 µl

RT (Reverse Transcriptase) Enzyme) was added and put in 43°C for 1 hour.
PCR was done using long expand PCR kit (Roche) as follows: 2,5 µl of RT product was added to 47,5 µl of mixed buffer (38,9 µl of diethyl pyrocarbonate (DEPC)-treated water, 4 µl Buffer1, 1,75 µl DNTPs, 0,8 µl of each Forward or Reverse primers and 0,75 µl of long expand polymerase) and put in thermocycler in following conditions 94°C for 2 minutes (1 cycle), 10 cycles in 94°C for 10 seconds, 55°C for 10 seconds, 68°C for 2 minutes, 28 cycles in 94°C for 15 seconds, 55°C for 30 seconds, 68°C for 2min+20seconds cycle elongation for each successive cycle and finally 1 cycle in 68°C for 7 minutes. The long expand PCR products run on agarose gel (1%) and corresponding band revealed the BSBV-Ny isolate RNA1, RNA2 and RNA3 after staining by ethidium bromide (Fig. 11).

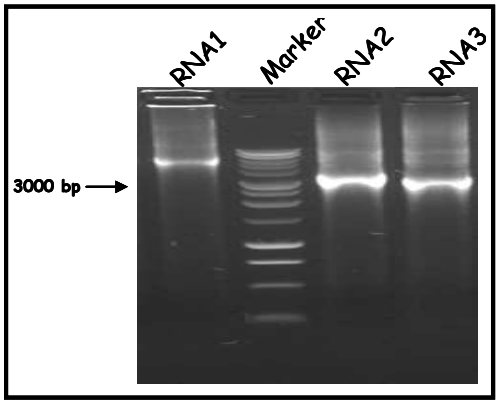


Fig. 11 Full-length fragment of RNA1, RNA2 and RNA3 of Iranian BSBV-Ny isolate from Iran (Nyshabour, Khorasan Razavi province)

X.3.2.2 Cloning of BSBV-Ny isolate full-length in pGMT vectors

Full-length of BSBV-Ny isolate was cloned in pGMET vectors. Inserted fragments selected and amplified in liquid LB medium (containing ampicilin) for 16 hours. The DNA extraction was done on plasmid vectors using Mini prep kit (Qiagen) (Fig. 12, 15).

X.3.2.3 *Beet soil-borne virus* RNA1, RNA2 and RNA3 restriction

KspI (SacII) and SpeI digested the full-length clones of RNA1 and NcoI and SpeI digested the full-length clones of RNA2 and RNA3 of BSBV-Ny constructs and liberated the clones inserted from the plasmid vector, providing a template from which transcription would initiate one G nucleotide before the viral sequence (Fig. 12).

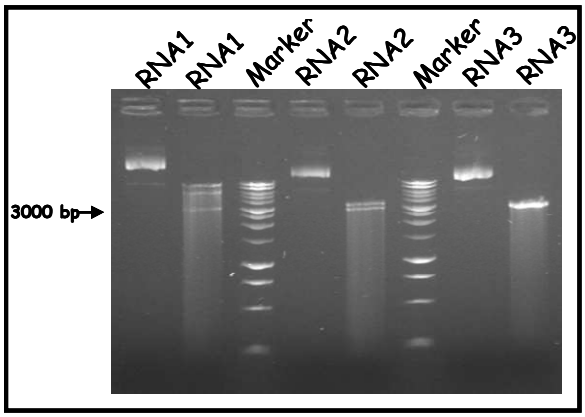


Fig. 12 Restriction of full-length fragment of RNA1, RNA2 and RNA3 of Iranian BSBV-Ny constructs by KspI, NcoI and SpeI restriction Enzymes

X.3.2.4 *Beet soil-borne virus* RNA transcriptions in vitro

Transcription reaction with T7 RNA polymerase for generating capped RNA as aforementioned was used to produce genome-length RNA for inoculation to plant leaves (*Chenopodium quinoa*).
Infectivity of the transcript RNA was tested by suspending 2 to 5 µg of RNA in GKP buffer (50 Mm glycine, 30 mM K2HPO4, 1% bentonite, and celite, pH 9.2) and mechanically inoculated on leaves of *C. quinoa* (Fig. 13) (Weiland & Edwards, 1994).

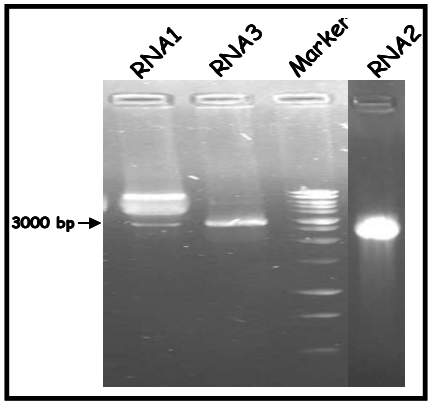


Fig. 13 Transcription of Iranian BSBV-Ny RNA1, RNA2 and RNA3 constructs by RiboMAX T7 kit.

X.3.3. Primers which used for Iranian BNYVV, BBSV, BBSV satellite, BSBV and BVQ detection, amplification and making full-length clones

Primer	Orientatio	Target	Sequence (5′-3′)	Accession	Nucleotide position on RNA
BNYVV2(4)F	Forward primer	RNA-2	CCATTGAATAGAATTTCACCGT	X04197	18-38
BNYVV2(4)R	Reverse primer	RNA-2	CCCCATAGTAATTTTAACTC	X04197	1069-108 8
BNYVV2(1)F	Forward primer	RNA-2	ACATTTCTATCCTCCTCCCAC	X04197	804–823
BNYVV2(1)R	Reverse primer	RNA-2	ACCCCAACAAACTCTCAAC	X04197	1330–1349
BNYVV2(7)F	Forward primer	RNA-2	AAGAACAGGAATTACGGTTGCG	X04197	1751-1772
BNYVV2(7)R	Reverse primer	RNA-2	CGCAACCGTAATTCCTGTTC	X04197	1753-1772
BNYVV2(8)F	Forward primer	RNA-2	CTACTGATAGCGGACCTCAC	X04197	1228-1247
BNYVV2(8)R	Reverse primer	RNA-2	ACCCCGTTCCATTTATACCC	X04197	2311-1992
BNYVV2P14(2)F	Forward primer	RNA-2	TATGGGGATGGTAGATAGTTT	X04197	4042-4062
BNYVV2P14(2)R	Reverse primer	RNA-2	TTGTCGATCCTGAGGTGTAA	X04197	4407-4426
BNYVV3(1)F	Forward primer	RNA-3	CAGTTTATGATTTAGGGCACA	M36894	465–486
BNYVV3(1)R	Reverse primer	RNA-3	ATCATCATCAACACCGTCAG	M36894	1081–1100
BNYVV3(2)F	Forward primer	RNA-3	GTGATATATGTGAGGACGT	M36894	51-70
BNYVV3(2)R	Reverse primer	RNA-3	CCGTGAAATCACGTGTAGTT	M36894	1250-1269
BNYVV4a1F	Forward primer	RNA-4	GGATCCAGTCTATCAGTAAGGGGTA	AF197554	288-312
BNYVV4a2R$_{his}$R	Reverse primer	RNA-4	TCGTTCACAAGCCTCCTTG	AF197554	1268-1286

Table 2 The oligonucleotide primers used in BNYVV RNA2, 3 and 4 RT-PCR amplification

Primer	Sequence (5′-3′)	Accession	position	
BNYVV2-1F	(Kruse *et al.*, 1994)	X04197	18-38	
BNYVV2-1R	(Kruse *et al.*, 1994)	X04197	1069-1088	
BNYVV2-2F	(Meunier, 2005)	X04197	804–823	
BNYVV2-2R	(Meunier, 2005)	X04197	1330–1349	
BNYVV2-3F	(Meunier, 2005)	X04197	1751-1772	
BNYVV2-3R	(Meunier, 2005)	X04197	1753-1772	
BNYVV2-4F	(Saito *et al.*, 1996)	X04197	1228-1247	
BNYVV2-4R	(Saito *et al.*, 1996)	X04197	2311-1992	
BNYVV2-5F	TATGGGGATGGTAGATAGTTT	X04197	4042-4062	This study
BNYVV2-5R	TTGTCGATCCTGAGGTGTAA	X04197	4407-4426	This study
BNYVV3-1F	(Meunier, 2005)	M36894	465–486	
BNYVV3-1R	(Meunier, 2005)	M36894	1081–1100	
BNYVV3-2F	(Kruse *et al.*, 1994)	M36894	51-70	
BNYVV3-2R	(Kruse *et al.*, 1994)	M36894	1250-1269	
BNYVV3-3F	(Koenig *et al.*, 2005)	M36894	325-347	
BNYVV3-3R	(Koenig *et al.*, 2005)	M36894	1198-1180	
BNYVV4-1F	GGATCCAGTCTATCAGTAAGGGGTA	AF197554	288-312	Legrève, unpublished
BNYVV4-1R	TCGTTCACAAGCCTCCTTG	AF197554	1268-1286	Legrève, unpublished

Table 3 The oligonucleotide primers used in BNYVV RNA2, 3 and 4 RT-PCR amplification

Primer	Sequence (5′-3′)	Accession	position	
BBSV(1)F	ATTAGATCCCACATCCTGGTGTGGTTAATC	AF452884	3341-3360	Mehrvar, unpublished
BBSV(1)R	GGGCACCTGGAAGACCAGGTATATAAG	AF452884	3624-3644	Mehrvar, unpublished
BBSV(2)F	TGATCAGCGATCATGGATTC	AF452884	24-43	Mehrvar, unpublished
BBSV(2)R	AGGTCATGAAACCGGGATAA	AF452884	909-928	Mehrvar, unpublished
BBSV(3)F	AGGGCCCTCATATGGAAAAT	AF452884	626–645	Mehrvar, unpublished
BBBS(3)R	TGATGTCCCCAGACATACGA	AF452884	1508–1527	Mehrvar, unpublished
BBSV(4)F	ATGCTTGGCTTAGCACGTTT	AF452884	1060-1079	Mehrvar, unpublished
BBSV(4)R	AGGGGAAACTGCATGAAGAA	AF452884	1938-1957	Mehrvar, unpublished
BBSV(5)F	CTCAGACACGCTGAGGAACA	AF452884	1179-1198	Mehrvar, unpublished
BBSV(5)R	AATGAGTAGCGGGATTGTGG	AF452884	2061-2080	Mehrvar, unpublished
BBSV(6)F	TTGCCAACAATGGTGATGAT	AF452884	1609-1628	Mehrvar, unpublished
BBSV(6)R	ACAATTGCAACAACCAATGC	AF452884	2472-2491	Mehrvar, unpublished
BBSV(7)F	TGTAGGGCAATCTGCTGTCA	AF452884	2035–2054	Mehrvar, unpublished
BBSV(7)R	TTGCAGTGAGTGCGGTAATG	AF452884	3192–3211	Mehrvar, unpublished
BBSV(8)F	AAGAAACCTAACCAGTTTCTCGTTG	AF452884	1-25	Mehrvar, unpublished
BBSV(8)R	GGGCACCTGGAAGACCAGGTATATAAG	AF452884	3624-3644	Mehrvar, unpublished
BBSV(10)F	GGGTCCATCGGTTTCTATGA	AF452884	2353-3372	Mehrvar, unpublished
BBSV(10)R	CAGACAGGTGCTGTGATGCT	AF452884	3049-3068	Mehrvar, unpublished
BBSV(12)F	GTACGCGTGTCACCAACACT	AF452884	2777-2796	Mehrvar, unpublished
BBSV(12)R	CCCCCAGCTATGATAGGACA	AF452884	3579-3598	Mehrvar, unpublished
BBT75fwd	Weiland et al. (2007)			
BBSVsat4F	GAAAGCTACGTGGTCATGGT	NC_006460	1-20	Mehrvar, unpublished
BBSVsat4R	GGGTAGCAATTAATTGCTTT	NC_006460	596-615	Mehrvar, unpublished

Table 4 The oligonucleotide primers used for sequencing and making full-length amplification of different Iranian BBSV and BBSV satellite isolates.

Primer	Sequence (5′-3′)	Accession	position	
BSBV(1)F	TAATACGACTCACTATAGGTATTATTTTCTCAACGGTTAT	**NC_003520**	**1-22**	**Mehrvar, unpublished**
BSBV(1)R	GGGCTTGATTTCCCTCAAGTAAAGGGG	**NC_003520**	**5834-5809**	**Mehrvar, unpublished**
BSBV(2)F	TAATACGACTCACTATAGGGTAATTTTACTTCTTCGCCGT	**NC_003518**	**1-22**	**Mehrvar, unpublished**
BSBV(2)R	GGGCTTGATTTCCCTCAAGTTAAGGGG	**NC_003518**	**3454-3427**	**Mehrvar, unpublished**
BSBV(3)F	TAATACGACTCACTATAGGGTATTTTTTTTTCTTGCCGGT	**NC_003519**	**1–22**	**Mehrvar, unpublished**
BSBV(3)R	GGGCTAGATTTCCCTCTAGTTAAGGGG	**NC_003519**	**3005–2978**	**Mehrvar, unpublished**

Table 5 The oligonucleotide primers used for making full-length construct of Iranian BSBV-Ny isolate.

Primer	Sequence (5′-3′)	Accession	position
BVQ1(1)F	(Meunier *et al.*, 2003)	AJ223596	2526-2547
BVQ1(1)R	(Meunier *et al.*, 2003)	AJ223596	2817-2796
BVQCPfor	CCATGGTTGATCCAAGATATGA	NC_003511	326-347
BVQCPrev	CTATGAGCCGGTCCACTTCAAT	NC_003511	825-804

Table 6 The oligonucleotide primers used for detection and sequencing of Iranian BVQ from different part of the country.

X.3.4. Vectors which used for full-length cloning and sequencing of Iranian BNYVV, BBSV, BBSV satellite, BSBV and BVQ

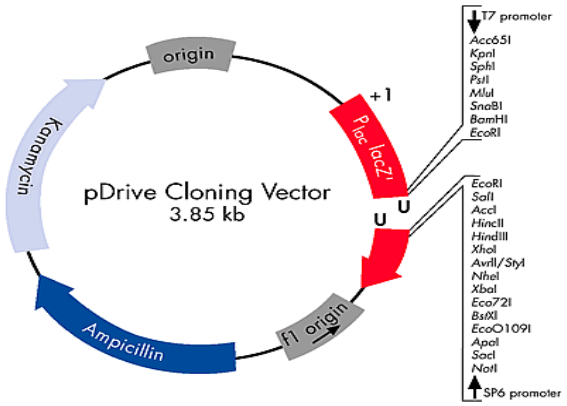


Fig. 14 pDrive Vector (Qiagen)

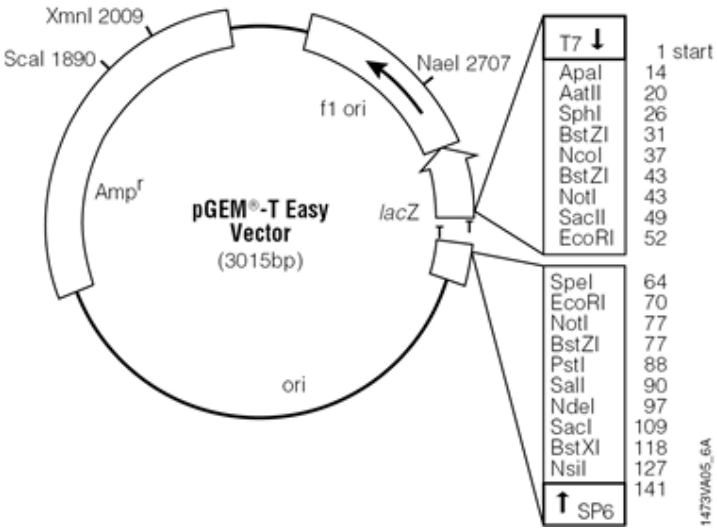


Fig. 15 pGEM-T Easy Vector (promega)

X.4. Name and place of collected sugar beet samples from Iran (Sep. 2006)

No	Place of gathering and name of the farmers
1	Chenaran(Mochenan)
2	Chenaran(Mochenan)
3	Chenaran(Mochenan)
4	Chenaran(Mochenan)
5	Ghochan
6.	Nyshabour (Jomhouri village) the same field of no. 35
7	Torbat Hydarieh (Jolgeie Rokh)
8	Torbat Hydarieh (Jolgeie Rokh)
9	Torbat hydarye(TH1)
10	Marvdasht (Koshk)
11	Biza(Satalan village shahram zare) extraction
12	Ramjerd (Abas Abad gholam hosein jafari)
13	Marvdasht (Koshk)
14	Marvdasht (Koshk)
15	Ramjerd (Abas Abad)
16	Fars-Biza
17	Fars-Biza
18	Zarghan
19	Zarghan
20	Mashhad (Ferdowsi) Haji hydari
21	Shirvan (Baghan 1)
22	Chenaran (Sayed Abad)
23	Shirvan (Baghan 2)
24	Bojnourd (Gharlegh 3)
25	Chenaran (Radkan 1)
26	Ghochaqn (Almajogh)
27	Shirvan (Power Plant Field)
28	Bojnourd (Gharlegh) Mrs. Gharoni sample
29	Bojnourd (Garmkhaneh 1)
30	Shirvan (Baghan 3)
31	Bojnourd (Gharlegh 2)
32	Shirvan (Baghan4)
33	Nyshabour (Shadiakh)
34	Nyshabour (Jomhouri2) leaf extraction
35	Nyshabour (Jomhouri3) root extraction
36	Nyshabour (Jomhouri4) (good sequence of P25 in For) chenopodium
37	Sabzevar (Sayed Abad1) Rajab Ali Sayed Abadi
38	Sabzevar (Sayed Abad2) Noorolah Sayed Abadi
39	Sabzevar (Soltan Abad1) Ali Reza Biabani

40	Chenaran (chahchah) Farkhondeh Sadat
41	Sabzevar (Soltan Abad2) Ali Akbar Namani
42	Chenaran (Kalate Sado) Aiobi
43	Nishabour (Biabani)
44	Nishabour (Chakane) Arazi abdolah Give
45	Mashahd (Ahmad Abad)
46	Fariman (7 Hectares)
47	Fariman (Namazi 20hectare)
48	Fariman (Mrvi 15 Hectare)
49	Torbat Jam (Momen Abad 2.5 Hectare)
50	Torbat Jam (Noor Abad Mr. Dehghan 3h)
51	Mashahad (Shir Hesar1)
52	Mashahad (Shir hesar2 Mr. Javad Alirezaei)
53	Chenaran
54	Torbat Jam (Khyr Abad 2, Mr. Mohammad Dshti 3h)
55	Nyshabour (Shahar Kohne, Hemat Abad Village Mr. Barat Ali Garmabi)
56	Nyshabour (Shahre Kohne, Khyr Abad district)
57	Shirvan(Baghan5)SHB9
57-2	Chenaran (Gahveh) Mr. Rhimzadeh
58	Chenaran(M1)
58-2	Chenaran (Gahveh) Mr. Rhimzadeh
59	Boujnord(Gharlegh4)BGH9
60	Boujnord(Gharlegh5)BGH
61	Torbat Hydarieh (Nazar Abad3) GNA-3
62	Sabzevar (S2)
63	Torbat Hydarieh (Nazar Abad) GNA-4
64	Sabzevar (S3)
65	Sabzevar (S1)
66	Golgei Rokh(A-2)
67	Torbat Hydarieh2
68	Mashhad 1, IPPO
69	Fariman (Janb khane behdasht kar)IPPO
70	Chenaran (Hakim Abad 1, After Azar Kar, 10H) IPPO
71	Mashhad (JADE Khoram, Nazeran Field,) IPPO
72	Fariman (Khyr Abad, Mr. Ghorban Farhadi, 8H) IPPO
73	Chenaran (Akhlamad 1, After, 20H), IPPO
74	Fariman (Shahan Garmab road, Atar field, 10H) IPPO
75	Fariman (Ghalandar Abad 1 Mr. Farimanian, 5H) IPPO
76	Chenaran (Akhlamad 2 before paste factory) IPPO
77	Chenaran (Hakim Abad 2, before sale Abad, 4H) IPPO
78	Mashhad (Before agor feshari kore akhtar nia, Mr. Esfandiari) IPPO
79	Fariman (Rezaei, well no 3) IPPO
80	Fariman (Ghalandar Abad 2, Mr. Pori well , 20H) IPPO

81	Torbat jam 2
82	Fariman (Ghalandar Abad 3, Mr. Farimanian, 2H) IPPO
83	Chenaran (Radkan 2 Road, 15H) IPPO
84	Fariman (Mr. Behzadian, well no, 1) IPPO
85	Chenaran (Hossein Abad, Mr. Taheri, 4H) IPPO
86-1	Fraiman (Behzadian, Well no,7 -7H) IPPO
86-2	Fraiman (Behzadian, Well no,7 -7H) IPPO
86-3	Fariman (Behzadian, well no. 7)
87	Torbat Jam (Khyr Abad 2, Mr. Ali jan, near well, 5H) IPPO
88	Chenaran (before the city, against alghadir complex, 3H) IPPO
89	Torbat jam (Rkhneh village, 10H, Mr. Yousofi) IPPO
90	Chenaran (before 3 km, 7H) IPPO Good sequence in Rev.
91	Torbat Jam (Before Nasr Abad 1, Mr. Asghari, 1H) IPPO
92	Fariman (Safi Abad, Mr. Ghodrat o lahe Khjezadeh) IPPO
93	Fariman (Behzadian, well no, 2, 10H) IPPO
94	Torbat Jam (Before Nasr Abad 2, 10H) IPPO
95	Chenaran (After, Mr. Rezaei field, 7H) IPPO
96	Torbat Jam (Jim Abad Razavieh district, Mr. Mohammad Etebari) IPPO
97	Nyshabour (Shahre Kohne, Mr. Hemati)
98	Nyshabour (Tahte Jolge, Mr. Mohammad Bagher nejad) IPPO Good sequence in Rev.
99-1	Nyshabour (Tahte Jolge, Mir Abad village 1, Mr. Barat Ali Garmabi) IPPO
99-2	Nyshabour (Tahte Jolge, Mir Abad village 2, Mr. Barat Ali Garmabi+ Abas ali) IPPO
100	Sharoud (Myamei, Ebrahim Abad
101-1	Sharoud (Ghale Zendani),
101-2	Sharoud (Ghale Zendani), Chenopodium
103	Hamadan(Malayer-avard Zaman, Noshijan)
104	Hamadan(Nahavand, Gian Village1, Mirza Morad Giani)
105	Hamadan(Firozan, Sade Vaghas Village)
106	Hamadan(Nahavand, Gian Village 2)
107	Hamadan(Asad Abad, Lak Lak Village)
108	Hamadan(Malayer-Dehno Avard Zaman, Amire Rostami)
109	Hamadan(Asad Abad, Mosi Abad Village1)
110	Hamadan(Asad Abad, Mosi Abad Village2)
111	Hamadan(Malayer, Hezar Jerib Village)
112	Hamadan(Firozan, In front of filling station field)
113	Bokan (Ghazman, Mr. Abobakr Ebrahimi)
114	Miandoab (Haji Behzad1, Markazi district, Mr. Mohammad Teghi field)
115	Miandoab (Koh Khan, Mr. Esmaeil Tarkhini field)
116	Miandoab (Haji Behzad2, Markazi district, Mr. Feridon Nasirian field)
117	Oromieh, Mohammad Yar1
118	Khoy (Outskirt of city)
119-1	Salmas (Pikjik Village1, Mr. Hamid Vare)
119-2	Salmas (Pikjik Village, Mr. Hamid Vare) Chenopodium

120	Mohammad Yar2
121	Bokan (Oshi Tape Village, Mr. Rahim Eshgi field)
122	Oromieh, Mohammad Yar3 (Close to Oromieh Salt Lake)
123	Salmas (Pikjik Village, Mr. Hassan Khajeh Loo)
124	Miandoab (Kholahar, Mr. Saeed Shikh Rezaei)
125	Khoy
126	Pars Abad (Iran Abad 1, Mr. Bavala field)
127	Bokan
128	Pars Abad(Sugar Plant)
129	Pars Abad(Before the city 5 km from Geremi)
130	Pars Abad (Agir loo Mr. Tarikhi field)
131	Pars Aba (Kesht o Sanat moghan, Section 3
132	Pars Abad (Iran Abad 2, A sample from IPPO ardabil)
133-1	Ardabil (5 Km before the city from Miane) Sugar beet
133-2	Ardabil (5 Km before the city from Miane) Chenopodium
134	Ardabil (Aghalam) IPPO sampleC
135	Pars Abad (Agir loo district)
136	Pars Abad (Agir loo district)
137	Kermanshah (Bistone, Pamchal Patapeh, dosti section)
138-1	Islam Abad Gharb (Sia Sia Chikheh Area1)
138-2	Islam Abad Gharb (Sia Sia Chikheh Area 2) chenopod
139	Islam Abad Gharb (Sia Sia Chikheh Area3)
140	Kermanshah (Bistone, before bazm abad)
141	Islam Abad Gharb (Sia Sia Chikheh Area4, Mr. Ghasem Khosravi)
142	Kermanshah
143	Kermanshah(Islam Abad Gharb, hamil area)
144	Kermanshah, Abas Abad, Mr. Ahmad Jamali, 2H)
145	Kermanshah (Rostam Abad, Mr. Vali Mehrianher)
146	Kermanshah (Bistone, Bazm Abad village, Mr. Khosravi)
147	Kerman shah (Biseton)
148	Kermanshah(Dolah, Mr. R. Gh. Dolaei)
149	Kermanshah (Bistone, 13) Basati
150	Kermanshah (Central District, Mr. Abdolahi & Fatahi) Basati
151	Kermanshah (Unknown)
152	Kermanshah (15) Basati
153	Kermanshah
154	Kermanshah (Kangavar, Taher Abad Village, Mr. Hossein Salehi)
155	Kermanshah (Kangavar 1)(Field in the ring of city toward Hamadan)
156	Azarbaeijan Sharghi(Azar Shahr, Malekan3)
157	Azarbaeijan Sharghi (Azarshahr & Malekan2)
158	Azarbaeijan Sharghi (Azarshahr & Malekan3)
159	Azarbaeijan Sharghi (Azarshahr & Malekan4)
160	Ilam(Shirvan Chardavol, Helidan district2)

161	Ilam(Shirvan Chardavol, Helidan district3)
162	Ilam(Shirvan Chardavol, Shabab Village)
163	Kerman (Bardsir, Dashtkar Area 1)Motor pomp shahid Behshti 1
164	Kerman (Bardsir, Dashtkar Area 2)Motor pomp shahid Behshti 3
165	Kerman (Bardsir, Dashtkar Area 3)Motor pomp shahid Behshti 2
166	Ghazvine (Boein Zahra, Amin Abad Kohneh, 11 Km before boein zahra from ghazvin, , Mr Mohammad Rezapour)
167	Ghazvine (Boein Zahra, Amin Moket Road Mr. Younos Bahrami)
168	Ghazvine (pour Yousufian Lob Field, Mr. Ali Sadeghi)
169	Ghazvine (Boein Zahra, Mr. Haj Taghi Abdol Hosseini)
170	Ghazvine (Boein Zahra, Amin Moket Road Mr. Roholah Ghanei)
171	Torbat Jam (Momen Abad 2 village, 40 Km after Torbat Jam, 15H, Plant Protection Organization Collected Samples)
172	Mashhad (Ahmad Abad, Gandoraz Village)
173	Nghab
174	shirvan
175	Sabzvar
176	Shirvan (Field beside Faculty of Agriculture)
177	Mashhad (Sad Abad Torkan Village)
178	Torbat Jam (Paein Jam District, Mr. Mohammad Rahmati, 2H, PP)
179	Golgei Rokh1
180	Islam ghale
181	Fariman (Mahdawi Well, 25 Hectares)
182	Golgei Rock (GRA-4) Ahmad Abad
183	Golgei Rock (GRA-12) Ahmad Abad
184	Golgei Rock (Well No. 4) (GR-1)
185	Golgei Rock (Well No. 4) (GR-2)
186	Golgei Rock (Well No. 4) (GR-4)
187	Golgei Rock (Well No. 4) (GR-5)
188	Golgei Rock (Well No. 4) (GR-6)
189	Golgei Rock (Well No. 4) (GR-7)
190	Golgei Rock (Well No. 4) (GR-10)
191	Golgei Rock (Well No. 4) (GR-12)
192	Golgei Rock (Well No. 4) (GR-13)
193	Golgei Rock (Mohandes Nabavi) (GNB-2)
194	Golgei Rock (Mohandes Nabavi) (GNB-3)
195	Golgei Rock (Mohandes Nabavi) (GNB-5)
196	Golgei Rock (GNA-6) Nazar Abad
197	Golgei Rock (GNA-7) Nazar Abad
198	Golgei Rock (GNA-8) Nazar Abad
199	Golgei Rock (GNA-9) Nazar Abad
200	Golgei Rock (GNA-10) Nazar Abad
201	Golgei Rock (GNA-12) Nazar Abad

202	Golgei Rock (GNA-13) Nazar Abad
203	Golgei Rock (GNA-14) Nazar Abad
204	Robat sefid
205	Torbat Hydarieh (TH2)
206	Pars abad moghan
207	Pars abad moghan
208	Pars abad moghan

Soil samples from sugar beet fields in Iran

1	Hamadan(Nahavand, Gian Village1, Mirza Morad Giani)
2	Kermanshah (Rostam Abad, Mr. Vali Mehrianher)
3	Islam Abad Gharb (Sia Sia Chikheh Area1)
4	Kermanshah(Dolah, Mr. R. Gh. Dolaei)
5	Kermanshah (Unknown)
6	Kermanshah
7	Ilam(Shirvan Chardavol, Shabab Village)
8	Hamadan(Nahavand, Gian Village 2)
9	Ramjerd (Abas Abad gholam hosein jafari)
10	Zarghan
11	Zarghan
12	Ramjerd (Abas Abad)
13	Kermanshah (15) Basati
14	Kerman (Bardsir, Dashtkar Area 2)Motor pomp shahid Behshti 3
15	Kerman (Bardsir, Dashtkar Area 3)Motor pomp shahid Behshti 2
16	Ghazvine (Boein Zahra, Amin Moket Road Mr. Younos Bahrami)
17	Marvdasht (Koshk)
18	Marvdasht (Koshk)
19	Marvdasht (Koshk)
20	Fars-Biza
21	Fars-Biza
22	Hamadan(Malayer, Hezar Jerib Village)
23	Ilam(Shirvan Chardavol, Helidan district3)
24	Ghazvine (pour Yousufian Lob Field, Mr. Ali Sadeghi)
25	Ghazvine (Boein Zahra, Amin Moket Road Mr. Roholah Ghanei)
26	Torbat Jam (Paein Jam District, Mr. Mohammad Rahmati, 2H, PP)
27	Kermanshah (Bistone, Bazm Abad village, Mr. Khosravi)
28	Kermanshah (Bistone, before bazm abad)
29	Kermanshah (Bistone, 13) Basati
30	Kermanshah (Central District, Mr. Abdolahi & Fatahi) Basati
31	Azarbaeijan Sharghi(Azar Shahr, Malekan3)*
32	Islam Abad Gharb (Sia Sia Chikheh Area4, Mr. Ghasem Khosravi)

33	Kermanshah (Kangavar, Taher Abad Village, Mr. Hossein Salehi)
34	Biza(Satalan village shahram zare) extraction
35	Kermanshah(Islam Abad Gharb, hamil area)
36	Bokan (Ghazman, Mr. Abobakr Ebrahimi)
37	Miandoab (Koh Khan, Mr. Esmaeil Tarkhini field)
38	Ghazvine (Boein Zahra, Amin Moket Road Mr. Younos Bahrami)
39	Hamadan(Asad Abad, Lak Lak Village)
40	Hamadan(Asad Abad, Mosi Abad Village2)
41	Hamadan(Firozan, In front of filling station field)
42	Kermanshah (Bistone, Pamchal Patapeh, dosti section)
43	Kermanshah
44	Kermanshah, Abas Abad, Mr. Ahmad Jamali, 2H)
45	Kermanshah (Kangavar 1)(Field in the ring of city toward Hamadan)
46	Fariman (Behzadian, well no, 2, 10H) IPPO
47	Fariman (Safi Abad, Mr. Ghodrat o lahe Khjezadeh) IPPO
48	Fariman (+)(Namazi 20hectare)
49	Fariman (Behzadian, well no. 7)
50	Azarbaeijan Sharghi (Azarshahr & Malekan4)
51	Mashhad (Ahmad Abad, Gandoraz Village)
52	Miandoab (Haji Behzad2, Markazi district, Mr. Feridon Nasirian field)
53	Golgei Rock (Well No. 4) (GR-1)